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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

I. The Spontaneous Tumor and Associated Abnormalities.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 31 to 40.

(Received for publication, December 11, 1922.)

The frequency with which malignant tumors develop at the site of old syphilitic lesions or in association with long standing inflammatory processes of a syphilitic nature, has led many to believe that there may be some etiologic connection between syphilis and malignant disease. However, it is still uncertain whether syphilis, as such, can be regarded as a determinative factor in the incidence of malignant growths or whether its influence is confined to the realm of chronic irritation. Until recently, no experimental investigation of the relation existing between syphilis and malignant disease was possible, due to the lack of an animal in which both conditions could be studied experimentally. This obstacle has now been removed by the occurrence of a highly malignant tumor in a rabbit infected with syphilis, and the successful transplantation of the tumor to other rabbits, a brief report of which was made in March, 1921 (1).

The tumor has been under observation for more than 2 years and is now (November, 1922) in its twentieth generation. During this time, a careful study of the tumor has been made and it has been employed in an extensive series of experiments, the immediate object of which was to determine whether any essential connection could be established between syphilitic infection and the occurrence of malignant growths. The study of this problem has necessitated a collateral investigation of a wide range of conditions including circumstances affecting the growth or malignancy of the tumor, the effects of the tumor upon the animal organism, the influence of syphilitic infection upon resistance to tumor growth and upon an established tumor immunity, and the converse relationship of the effect

of tumor inoculation upon the course of a subsequent infection with *Treponema pallidum*; finally, through the joint use of the two experimental agencies (syphilis and tumor), controlling the one by the other, information has been acquired concerning the mechanism of animal resistance to syphilis as well as to the growth of the tumor.

So little is known of malignant tumors of the rabbit and of pathological conditions which may be associated with them, that it seems advisable to preface the reports of our experimental work by a detailed account of the tumor itself and of other abnormalities present in this animal. In the descriptions which follow it may appear that too much space has been devoted to what might be regarded as uninteresting detail or conditions of little or no importance. It seems well to state, therefore, that this treatment of the subject is intentional and is designed in part as a means of giving a comprehensive picture of the disease present in this animal, but its chief object is to place due emphasis upon a group of conditions which when properly correlated may prove to be of great value in interpreting phenomena of tumor growth. In fact, as will appear in subsequent papers, a great deal of the experimental work which has been carried out with this tumor has been based upon an interpretation of the relation existing between these conditions and the origin and malignancy of the tumor.

Many benign tumors have been reported in rabbits, but malignant growths appear to be extremely rare and in only three instances has successful transplantation been accomplished (2, 3) and all these tumors appear to have been lost before any considerable experimental work could be carried out. In our experience with rabbits, we have observed four instances of spontaneous tumors, three of which were in animals infected with *Treponema pallidum*. Three of the four growths were adenomata of the kidney encountered in the course of routine autopsies. The fourth tumor, which forms the subject of this paper, developed at the site of a scrotal chancre and was first noted 4 years and 3 months after inoculation. The tumor is unique in that a complete history of the animal is available, including photographic records of the syphilitic lesions, from the time of inoculation with *Treponema pallidum* to the occurrence of the tumor, with a subsequent history covering the progress of the growth over a period of 6 months. The essential features of this history will be summarized as briefly as possible.

Clinical History.

The animal was a male albino of a stocky build, weighing 1,900 gm. and fully developed when first placed under observation.

Inoculation and Development of Scrotal Lesions.—On June 11, 1916, the animal was inoculated in both scrota with the Zinsser-Hopkins strain of *Treponema pallidum* by the subcutaneous implantation of a bit of infected testicular tissue. Chancres developed in both scrota but completely disappeared during the summer.[1] 5 months after inoculation, there was a recurrence of the lesion in the left scrotum which assumed the form of a circumscribed but rather irregular nodular mass involving the deeper layers of the skin and subcutaneous tissue. This nodule increased slowly, reaching a maximum size of 1.9 by 1.5 by 1.8 cm. at the end of 3½ months. It then began to soften at the center and resolution took place without perforation of the skin. Resolution was slow and interrupted by intervals of slight renewal of activity. 18 months after inoculation, a small opaque mass was still present in the scrotum. Meantime, lesions had developed in both testicles with the production of a slight fibrous thickening and some atrophy which was more marked on the left than on the right.

Late Secondary Lesions.—Nothing more of importance was noted until April, 1919 (2 years and 10 months after inoculation), when a series of cutaneous lesions developed along the outer sides of both hind feet and disappeared within a few weeks. These lesions presented the appearance of small oval or flattened papules with scaly surfaces. On June 10, there was a slight iritis in the right eye, and in November, there was a recurrence of the cutaneous lesions on the feet.

Development of Chronic Inflammatory Reaction in the Left Scrotum.—In January, 1920, a chronic inflammatory condition developed in the left scrotum which probably marked the beginning of the series of local changes that culminated in the development of the tumor. The condition present is shown in Fig. 1, and may be described as a diffuse infiltrative process producing a parchment-like thickening of the skin over a large part of the ventral surface of the scrotum with desquamation and exfoliation of the epithelium over the center of the lesion. At first, the affected area was pale and translucent with a smooth and glistening surface. As the condition progressed, delicate silvery scales began to form and eventually the central part of the lesion became covered by thin crusts, the removal of which left moist or weeping patches. An attempt to demonstrate spirochetes in the lesions by dark-field examination gave negative results. The subsequent history of this affection was one of improvement or complete recovery followed by relapse.

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[1] Seasonal conditions exercise a marked influence upon the course of syphilitic infections in the rabbit. During the summer months, there is rarely the same degree of regularity in the course of the disease as at other seasons, while the early lesions tend to be slight and of short duration followed by relapse during the fall or winter months.

Papillomatous Affection of the Lips.—In March of the same year, small patches of a peculiar papillomatous growth appeared on the skin surface of the left upper lip near the midline, over the chin, and on the right lower lip toward the angle of the mouth. The growth was composed of soft thread-like processes of a grayish white or pale yellow color averaging about 2 to 3 mm. in length. They were firmly united to the skin and reminded one of soft stubby hairs, markedly enlarged. This condition persisted for some time with no tendency to spread but had almost disappeared at the time the animal was killed.

Demonstration of the Persistence of the Syphilitic Infection and Appearance of a Tumor Nodule in the Scrotum.—In September, 1920, there were no lesions visible, except an area of slight infiltration in the left scrotum, the surface of which was covered with fine scales. At this time, test inoculations made from a popliteal node showed that the animal still harbored viable treponemata (4).[2] On October 1, a small, translucent nodule with smooth and glistening surface was noted in the left scrotum; its position corresponded with that of the primary syphilitic lesion and with the upper portion of the area previously affected by the eczematoid condition described. This nodule grew rather rapidly but irregularly, and at one time, following aspiration with a needle, diminished greatly in size. On October 18, the nodule measured 1 by 0.8 by 0.2 to 0.3 cm. The appearance and mode of growth of the lesion closely resembled those of an active syphilitic granuloma, but several attempts to demonstrate spirochetes by dark-field examination gave negative results; hence, it was excised under ether anesthesia for histological examination. The appearance presented at this time is shown in Fig. 2. This lesion will be referred to subsequently as the primary tumor. At this time, the animal weighed 2,500 gm. and was apparently in excellent physical condition, except for the presence of a peculiar diffuse thickening of the skin, the history of which may be given at this point.

Nodular and Diffuse Induration of the Skin.—Several months before the development of the tumor nodule in the scrotum, it was noted that the skin of the animal was becoming unusually thick, especially in the region of the shoulder girdle where we were accustomed to grasp the animal in removing him from the cage. No especial significance was attached to this condition until the fall of 1920 when the thickening became extremely marked and almost board-like over the region of the right shoulder (the area covered by the fingers in the act of grasping). The development of this condition corresponded roughly with the occurrence of the growth in the scrotum. In November, 1920, a number of small, sharply circumscribed and intensely indurated nodules were found in the skin over the back, the sides of the thorax, and in the cutaneous folds of the flanks. These nodules were imbedded in the deeper layers of the skin; in general, the overlying skin was pale with an irregular and scanty growth of hair, while in places the surface was smooth and glistening. Associated with these lesions, there were irregular areas

[2] Brown and Pearce (4), p. 3, Rabbit 1.

of thickening in the skin such as occur during the shedding period. Some of the nodules ultimately disappeared, while others persisted until the death of the animal.

Reinoculation and Cross-Inoculation.—On October 28, 1920, the animal was reinoculated in the right testicle and on the sheath with the strain of *Treponema pallidum* recovered from its own popliteal lymph node. The reaction in the skin was very slight but was decidedly more marked in the testicle, lasting for 11 days before it began to recede. On November 15, a cross-inoculation with the Nichols Strain V was done in the skin at the base of the right ear and on the sheath. The reaction on the sheath was again slight but that on the ear was quite marked and assumed the form of a spreading infiltration with a scaly surface which persisted for 10 days and then gradually disappeared.

First Tumor Recurrence.—After excision of the primary growth, the wound in the scrotum healed quickly, leaving a thin and delicate scar. 1 month later, a tiny flattened nodule, identical with the one described above, appeared at the lower end of the scar, but soon disappeared. On December 28 (2½ months after excision of the primary growth), a second nodule was noted in the scrotum well above the area of the scar. This nodule grew rapidly with the formation of a superficial ulcer covered by a reddish brown crust as shown in Fig. 3. With the appearance of this lesion, the left inguinal nodes began to enlarge, reaching the condition seen in Fig. 3. A search for spirochetes in the cutaneous lesion was again made but with negative results. On January 13, 1921, the nodule in the skin and the nearest lymph node were removed under ether anesthesia (first recurrence).

Second Recurrence.—The wound healed slowly after this operation; a sinus formed which discharged a clear amber-colored and slightly oily fluid, but there was no suppuration. The upper lymph node continued to enlarge and there was an almost immediate recurrence of the growth in the skin. The process now assumed a more diffuse character and quickly extended across the midline in the pubic region; the nodes in the right groin began to enlarge and from this time onward there was a gradual loss of weight and strength.

Increased Activity of Growth and Physical Deterioration.—During February, there was a decided increase in the activity of the growth, which spread diffusely but irregularly over the pubic and lower abdominal regions, and by March 1, there was a decided deterioration in the physical condition of the animal. In addition to a slight loss of weight, and general weakness, there were marked weakness of the hind quarters, slight diarrhea, and relaxation of the anal and vesicle sphincters with dribbling of urine; the anus and sheath were swollen and reddened, and there was beginning ulceration along the mucocutaneous margins; the pupils were small (4 to 6 mm. in dim light) and irregular, and reacted feebly to light (flash) (spinal miosis.)

Terminal Condition.—By March 10, the animal was in extremely poor physical condition and was killed with ether. At this time there were extreme weakness and a loss of weight amounting to 300 gm.; the wounds were all clean but there was only slight union; the necrosis about the anus and sheath was spreading but the

tissues were comparatively dry and there was no appreciable inflammatory reaction; there was complete loss of sphincter control and movements of the hind legs were feeble, spastic, and incoordinate. Examination of the blood gave the following results.

Red blood corpuscles	3,040,000
White blood corpuscles	38,000
Hemoglobin, Sahli	80 per cent.
Palmer	60 per cent.
Differential count.	
Polymorphonuclear leucocytes	55 per cent.
Basophils	1 per cent.
Small mononuclears	20 per cent.
Large mononuclears and transitionals	24 per cent.

A few normoblasts were seen.

Transplantation.—Three attempts were made to transplant the growth to other rabbits. The first attempt was unsuccessful, but by the use of intratesticular inoculations, a good growth was obtained in both the second and third series of animals and subsequent transfers by this method have almost invariably given 100 per cent takes.

Pathology of the Excised Lesions.

Before proceeding with the report of the postmortem examination, which revealed the presence of a variety of conditions other than tumor metastases, it seems best to give a description of the lesions which were excised during life. For routine examination, these tissues, as well as others to be described, were fixed in Zenker's fluid and in formalin and stained with hematoxylin and eosin or with methylene blue and eosin.

Primary Growth.

The primary growth appeared as a small oval mass imbedded in the skin. It was firm in consistency, of a pale grayish white color, comparatively translucent, and fairly well demarcated from the surrounding tissue. Microscopically, this mass was composed of two distinct nodules (*A* and *B*, Fig. 4) separated from one another by a smaller nodule (*C*) which was continuous above with a narrow zone of growth in the papillary layer of the skin.

The largest and apparently the oldest of the nodules (*A*, Fig. 4) was located in the papillary layer of the skin and was surrounded on the lateral and lower surfaces by thin but compact bands of fibrous connective tissue (Fig. 4). This portion of the growth was made up of cells of an epithelioid type whose appearance and

arrangement are shown in Fig. 5 which was taken from the area designated as *a* in Fig. 4. Toward the periphery, the cells diminished in size, were round or oval in shape, and stained more intensely with hematoxylin.

The nodule as a whole was of a compact structure with only a slight connective tissue stroma and comparatively few blood vessels or lymph spaces. There were no areas of necrosis and very few cells showed mitotic division.

Toward the skin surface, the tissue was more loosely arranged than elsewhere, and as the epidermis was approached, gradually assumed the character of granulation tissue containing scattered islands and strands of epithelioid cells resembling those toward the center of the nodule (Figs. 6 and 7 from *b*, Fig. 4). This portion of the growth contained numerous dilated lymph spaces and thin walled vessels many of which showed a loose perivascular accumulation of lymphocytes. Capillary vessels were especially numerous immediately below the epidermis and in places appeared to invade the epithelial layer. At these places, there were numerous open spaces, some of which extended into the epidermis, as in Fig. 6, and were partly or completely filled with blood or with loose masses of epithelial cells, some of which were in process of division. In other instances, the appearance was that of an intracystic papilloma (Fig. 7). Many of these lesions bore a striking resemblance to those seen in Paget's disease. In this zone, there were also a number of irregular downgrowths from the surface epithelium, and these became more numerous toward the periphery, where they were continuous with a series of large papillary processes in the surrounding skin area (Fig. 4, left). Hair follicles were present in only a few places and these rarely extended to the surface. As a rule, they were atypical in appearance and grouped in nests with a slight zone of inflammatory reaction about them. There was unusually active mitotic division among the cells composing these follicles but little or no tendency to differentiation into the cortical substance of the hair.

Another feature of this nodule was the presence of scattered nests, or whorls, of cells resembling those of the surface epithelium (Fig. 5) and of multinucleated giant cells apparently composed of partially keratinized or degenerated cells.

Finally, the reaction about the deeper surfaces of the nodule was extremely slight; the connective tissue of the corium was reflected about it in the form of a capsule but there was very little cellular reaction.

The second nodule of the main tumor mass (*B*, Fig. 4) appeared as a bulbous expansion in the deeper layers of the corium, continuous with a smaller central nodule (*C*, Fig. 4) and with that portion of the growth lying immediately below the epidermis. It was partially enclosed on all sides by compact bands of fibrous connective tissue derived from the corium, while similar strands of tissue extended into the depths of the nodule. This part of the growth was composed of irregular round or oval cells with a granular or vacuolated cytoplasm, the outlines of which tended to be ragged and ill defined. Mitotic figures were numerous in all parts of the section as shown in Figs. 8 to 10 (from *c* and *e* of Fig. 4).

The structure of the growth also differed from that of the older nodule in that the cells were arranged in the form of a loose network enclosing irregular spaces or

vacuoles which apparently contained fluid or semifluid material of some kind. This produced a spongy appearance which can be recognized even in Fig. 4. The stroma consisted of clearly defined strands of connective tissue which followed the course of the larger vessels with no distinct network of intercellular fibrils (Figs. 8 and 9). There were numerous capillary vessels in all parts of the growth, with a system of prominent lymph spaces toward the periphery (Fig. 10).

The line of separation between the tumor cells and the connective tissue of the skin was not clearly defined. In places, there was a thick wall of fibrous tissue of almost normal appearance with very little cellular reaction (*d*, Fig. 4). At other points, the capsule was invaded for some distance by narrow columns of cells or was broken up into irregular strands by heavier masses of tumor growth. This condition was evident toward the upper surface, where columns of cells extended downward from the epidermis and joined with the mass in the corium (Fig. 4), but the break in the connective tissue was even more complete where nodules *B* and *C* united.

The extension of the growth in these directions also served to establish a connection with that part of the growth which was situated immediately below the skin surface. As shown in Fig. 4, the epidermis in general was greatly thickened and there were numerous irregular downgrowths of epithelium. These changes were associated with an active proliferation of the epithelial cells and in many places there was an outgrowth of cells which filled the intervening spaces of the papillary layer, as is indicated by the fading outlines of the process marked by an arrow in Fig. 10. This picture is characteristic of the condition present over the greater part of this area and is of considerable importance in tracing the origin of the tumor. As a rule, the growth of these cells was restricted to the loose connective tissue zone of the outer layer of the skin, but in a few places, as in Fig. 10, the cells penetrated to a greater depth. There was also a tendency to invade the lymphatics, and numerous tongue-like projections or masses of cells were found free in the lymph spaces of the tumor itself or in adjacent parts of the skin (Fig. 11 from *f*, Fig. 4).

It is also important to note that as the growth extended downward the appearance of the cells tended to change in one of two directions. Where there was obstruction to further growth, as along the line of contact with the dense fibrous layers of the corium, the cells became smaller, resembling the so called basal cells of the human epidermis, as may be seen in Fig. 12. Where less resistance was encountered, a transition to a more active type of cell occurred, as seen in Fig. 10, in which the growth has penetrated into the depths of the corium, forming the nodular mass previously described (*B*),

The remainder of the main tumor growth appeared as an irregular pyramidal mass (*C*, Fig. 4), wedged in between the two larger nodules *A* and *B*. An examination of a series of sections showed that this nodule might be regarded either as a downward extension of the growth in the papillary layer or as a part of nodule *B*. It was composed largely of cells of the type described in nodule *B* with a gradual transition toward the periphery into smaller cells of the basal type. In places,

these cells tended to be grouped about the lymphatics. Here again there were numerous polynucleated giant cell masses in the upper portion of the growth which appeared to be of epithelial origin as in the case of nodule *A*.

The skin about the central mass of tumor growth showed an interesting series of abnormalities which are believed to have a bearing upon the origin of the tumor. In general, there was a decided thickening of the epidermis with the development of large and irregular papillary processes. The cells were swollen and granular and many of them vacuolated, while mitotic figures were numerous in all parts of the epithelium.

The papillary layer of the skin was the seat of a low grade chronic inflammatory reaction which was peculiar in that the growth of capillary vessels was proportionately greater than that of the fibroblasts or the cellular infiltration. This reaction was present in all parts of the section but was more marked in some places than in others. The deeper layers of the skin were also abnormal. The individual fibers of connective tissue were distinctly swollen and irregular in outline and were collected into coarse bundles forming an irregular network or compact layers of highly refractile tissue which stained intensely with eosin. In places, there was an increase in the thickness of this portion of the skin. The walls of some of the vessels were also thickened and there were scattered patches of lymphocytic infiltration, some of which showed a perivascular arrangement.

There were practically no hairs projecting from this part of the skin and very few hair follicles were present. Some of these showed no appreciable abnormality, while others were in a state of active and somewhat atypical hyperplasia.

The most striking condition, however, was the presence of numerous areas of active and atypical epithelial growth which varied from a moderate hyperplasia to conditions such as those illustrated in Figs. 12 and 13 which are taken from different parts of the skin lying anterior to the area of the primary growth (*g*, Fig. 4). The majority of these lesions appeared to develop as downgrowths from hyperplastic papillary processes and tended to retain this form and structure as in Fig. 12. In other instances, the growth was more diffuse; the cells appeared to arise from a wider area and the outlines of processes tended to be obliterated. A third type of growth was that shown in Fig. 13, which is suggestive of a hair follicle, even the cross-sections presenting something of the appearance of a bulb, and occasionally, a clearly defined hair follicle formed the central structure in one of the lesions.

Other features of interest in these lesions are the variety of cells present, the transition from one type of cell to another, and the tendency in places to a perivascular arrangement, especially evident in the case of the grouping of the smaller cells about lymph spaces. It should also be noted that none of the lesions showed a tendency to prickle cell formation, and only one small area of keratinization was found in the entire area represented by the sections studied.

The downgrowth of epithelium in lesions of this type was usually arrested at the surface of the fibrous layer of the corium, as is clearly indicated in Fig. 12. In exceptional instances, however, the growth extended deeper as was the case with that in Fig. 13. The reaction about the lesions varied somewhat, but on the

whole, the growth of fibroblasts and cellular infiltration were less marked than is usual in simple chronic inflammatory conditions of the skin or in syphilitic infections, while the development of capillary vessels was nearer normal.

In portions of the skin still further removed from the primary growth, there were similar but less pronounced changes in both the epidermis and the subjacent tissues, the condition usually present being essentially that shown in Fig. 14.

Cutaneous Recurrences.

The first cutaneous recurrence consisted of a single large nodule with an ulcerated surface situated in the papillary layers of the skin and a number of smaller nodules imbedded in the fibrous layers. The structure of these lesions was almost identical with that of the most actively growing part of the primary tumor designated as nodule *B* (*B*, Fig. 4), except that the cells were even more irregular and less clearly defined and were much more loosely arranged. The growth was also more diffuse, and while in some places there was still a fair degree of separation between tumor and connective tissue, the blending of the two had become more complete; there was still a notable deficiency in the cellular reaction, and small columns and nests of tumor cells were widely distributed through adjacent parts of the corium.

In addition to the lesions described, the epithelium of the surrounding skin still exhibited an atypical hyperplasia. In some respects, the growth of epithelium was the same as that seen in wound healing or in many chronic inflammatory processes, but in places (Fig. 15), it was strongly suggestive of a primary malignant growth, the cells and character of the growth resembling that part of the primary tumor represented in Fig. 10. In other places, the most striking condition was an abnormal growth of hair follicles which resulted in the formation of giant masses such as that shown in Fig. 16. The cells of the mass were in a state of active division, and although hairs were developed, they were malformed and comparatively few of them appeared to reach the surface. In deeper parts of the skin, bulb-like structures were numerous, the majority being unusually large and containing many cells in process of division.

The second skin recurrence contained only a few small nodules analogous to those of the first, but was composed largely of a diffuse growth practically identical with that in Fig. 15 which appeared to arise directly from the surface epithelium and in places extended through the entire thickness of the skin with little or no reaction in the surrounding tissues.

The structural resemblance between successive cutaneous lesions is sufficiently close to identify them as parts of a common process; hence, the so called recurrent growths might be regarded either as direct extensions or as metastases from the original growth in the scrotum. Upon this basis, however, it would be difficult to explain the connection

which appeared to exist between the epidermis and the growth in the deeper layers of the skin in places far removed from the primary tumor. This feature of the lesions is more suggestive of the extension of some underlying condition or state such as favored the development of the primary tumor than of an extension of the tumor itself, a suggestion which is strengthened by the presence of a typical epithelial proliferations of a similar character in other parts of the body. These will be described later.

Metastases in the Inguinal Lymphatics.

The lymph nodes removed from the inguinal regions at the second and third operations were greatly enlarged, the capsules being thickened and of a dense fibrous consistency as shown in Fig. 17. The interior of the node was composed of a soft pasty material, somewhat slimy or cheesy in consistency, which tended to separate from the capsule. The color varied from an opalescent white to a grayish pink interspersed with areas of yellowish gray. In water, this material formed a flocculent suspension with only a few strands of fibrous tissue.

Microscopically, the parenchyma of the nodes was entirely replaced by a growth composed of cells of two main types, the character varying according to their distribution (Fig. 17). The cells toward the center of the growth were oval, rounded, or spindle-shaped with a granular and slightly basophilic cytoplasm. Toward the periphery, these were replaced by larger cells with a reticulated or even vacuolated cytoplasm which stained a pale pink or lilac color. Cells of this type were more numerous in the right inguinals than in the left, the growth on this side (right) being of more recent origin and more active, although mitotic figures were extremely numerous in all parts of both nodes. The framework of the lesions consisted chiefly of thin walled vessels some of which were distended with blood. These vessels were in striking contrast with those in the capsule and surrounding tissue which frequently showed great thickening of the walls and a narrow lumen. The connective tissue present was confined largely to the region of the vessels.

In the inguinal nodes, extensive areas of necrosis were seen for the first time (Fig. 17), associated with a distinct perivascular arrangement of the living cells, especially noticeable toward the center of the node.

Although the capsule of the nodes was greatly thickened, the connective tissue showed a distinct hyaline appearance with swelling or fragmentation of the fibers and was invaded by the tumor cells on all sides.

Autopsy.

At autopsy a variety of pathological conditions was found. In addition to metastatic growths in many organs, there were obvious

abnormalities of the skin, the tongue and buccal mucosa and esophagus, the cardiovascular system, the lymphoid tissues, the bones, the testicles, the thymus, the thyroid, the suprarenals, and the pituitary gland. For convenience, these conditions may be divided into two groups, the first including tumor metastases and associated reactions in affected organs, and the second, conditions which appear to have had some connection with the presence of the tumor or with the syphilitic infection or with both.

Metastases and Changes in the Affected Organs.

The organs or tissues in which tumor metastases were found included the lungs, the liver, the kidneys, the spleen, the bones, and the inguinal, the pelvic, and the retroperitoneal lymph nodes, and the inguinal fat. The general characteristics of the lesions were much the same in all organs, but the growth was most abundant in the liver, the spleen, and the bone marrow, while the liver contained the greatest variety of lesions. The liver will serve, therefore, as a basis for a general description of the tumor growth and the associated tissue reaction.

Liver.—The liver was approximately normal in size and of a yellowish brown color and contained numerous round or oval masses of a milky white or opalescent appearance, which varied from minute points to upwards of 1.5 cm. in diameter (Fig. 18). Most of these masses were sharply demarcated and of a firm elastic consistency, but the more translucent ones were soft and flaky and somewhat slimy to the touch. There was a prominent network of distended vessels about some of the nodules and a few showed small petechial hemorrhages or grayish yellow foci of necrosis. On the whole, however, the appearance was comparatively uniform, the chief difference being the degree of translucency or opacity. The liver was free from coccidiosis and no cysts were found in the abdominal cavity.

Histologically, there were innumerable areas of tumor growth ranging from a collection of a few cells in the capillary spaces with no reaction in the surrounding tissues to large nodular masses enveloped by a thick wall of connective tissue. Examples of these lesions are given in Figs. 19 to 21. The early lesions were composed of irregular round or oval cells with a granular and slightly basophilic cytoplasm. In most instances, these cells formed compact masses or solid cords which extended along the capillary spaces. Occasionally, however, the cells showed a radial arrangement about a central space containing albuminous material which suggested the appearance presented by a section of the deeper portions of a hair follicle.

The cells of older lesions differed greatly in appearance. The prevailing types were those seen in Figs. 19 to 21. Some nodules were composed entirely of small round or oval cells, as in Fig. 19, while others were made up of large, irregular, vacuolated cells such as those in Fig. 20. More often, there was an intimate combination of a variety of cells, as in Fig. 21, with the production of a lesion analogous to that of the primary tumor or a transition from one type of growth to another in different parts of the same nodule.

The structure of the growth in the liver was also subject to considerable variation. In general, the cells were arranged in the form of spongy or compact masses with comparatively little stroma, which was distributed chiefly along the course of the blood vessels. Many of the lesions preserved this structure throughout, while others showed a tendency to the formation of an irregular network of spaces toward the center of the nodule which were filled with a fluid or semifluid material. No intercellular network of connective tissue fibers could be demonstrated and only a few minute droplets of lipoid material could be brought out in the tumor cells by staining with Scharlach R.

The blood supply of some lesions was fairly abundant and there was frequently a tendency to a perivascular arrangement of the tumor cells. In such cases, the growth was usually composed of small round or oval cells with a granular cytoplasm. On the other hand, nodules composed of irregular, vacuolated cells contained fewer vessels as a rule and in extreme cases were practically devoid of a capillary network, the few vessels present being confined to stroma lines. This suggests that nutritive conditions were in part responsible for the cellular variations and that the vacuolation of cells was due to colloid or mucoid degeneration. This is borne out by the physical characteristics of these lesions (soft and slimy).[3]

Mass necrosis was present in only a few of the larger nodules and was confined to the center of the growth. Focal hemorrhages occurred under similar conditions.

The reaction about the liver metastases consisted chiefly in the development of connective tissue with very little cellular infiltration. The extent of this growth was as variable as that of the tumor itself. In fact, character of growth and extent of reaction appeared to be closely related. At one extreme, there was a group of lesions composed of small round or oval cells surrounded by a thick wall of fibrous connective tissue invaded to a greater or less extent by tumor cells. Fig. 19 was taken from the outer edge of a lesion of this type. At the other extreme, there were nodules, almost if not quite as large, made up of large vacuolated cells which showed little or no tendency to encapsulation, as seen in Fig. 20, and these two conditions were frequently found in close apposition to each other.

In any event, there were only a few instances of a clearly defined zone of connective tissue beyond which the tumor cells had not penetrated. As a rule, the surrounding connective tissue was diffusely invaded (Fig. 19) or was split up into a coarse network enclosing islands or nests of tumor cells which frequently extended

[3] Transplants made from lesions of this character were not so successful as those made from nodules composed of granular cells.

into the liver parenchyma beyond. Finally, some of the nodules showed concentric zones of tumor and connective tissue growth. The condition usually observed was that of a central mass of tumor cells surrounded by a more or less distinct zone of connective tissue followed by a second zone of tumor cells, then a zone of connective tissue, narrower than the first, and finally an outer zone of tumor growth diffusely invading the liver parenchyma. This condition is obviously suggestive of periods of increased activity on the part of the tumor cells or of decline in the resistance of the host and is comparable to the periodic growth frequently observed clinically in both man and animals.

The effect of the growth upon the liver itself was of two kinds. Apart from such effects as distortion of architecture and replacement of liver tissue by tumor growth, there was an advanced degeneration of liver cells associated with a moderate degree of hemosiderosis and necrosis of occasional cells or cell columns. The capillaries were moderately dilated but not congested. These conditions existed in parts of the liver which were not involved by the tumor growth.

A comparison of the growth in the liver with that in the scrotum or the inguinal lymph nodes is sufficient to indicate that the several groups of lesions were derived from a common parent cell. From the description of the growth in the liver and the reaction of the surrounding tissues, it is also apparent that differences in the structural characteristics of the lesions may be referred to such factors as age and rate of growth on the one hand, and on the other, to variations in the resistance encountered due to variations in functional capacity of the host.

Lungs.—Grossly, the lungs presented a normal appearance, except for the presence of numerous small opalescent or grayish white nodules distributed chiefly over the dorsal surfaces and through the substance of the two lower lobes. Microscopically, there were lesions of three types. The lymphatics and smaller blood vessels contained numerous collections of tumor cells with a granular, basophilic cytoplasm which formed lesions such as those in Fig. 22. In addition, there were larger nodules of growth composed of a mixture of granular and of vacuolated cells, while a few nodules were made up almost entirely of cells with a clear staining, vacuolated cytoplasm. Many of these lesions exhibited a peribronchial or perivascular arrangement. There was no reaction about the smaller pulmonary metastases and only a slight cellular infiltration in the alveolar walls surrounding the larger lesions.

Kidneys.—The kidneys presented an almost normal appearance. A single opalescent nodule measuring approximately 2 mm. in diameter was found in the inner portion of the cortex of one kidney. Microscopically, however, numerous collections of actively growing tumor cells were found in the capillaries of the glomerular tufts (Fig. 23), and in some instances, completely filled the glomeruli. A few lesions of similar character were present in the intertubular spaces of the cortex.

It is of interest to note that although there was no considerable tumor growth in the kidneys, there were well marked degenerative changes in both the tubular epithelium and the glomeruli, the latter condition manifesting itself as a hyaline thickening of the capillary walls (Fig. 23).

Spleen.—The spleen was a pale yellowish brown color, rather small, but distinctly swollen and contained five prominent nodules together with a number of smaller ones, which presented the same gross and microscopic appearance as those in the liver. In fact, the splenic pulp was largely replaced by tumor growth. The sinuses were filled with tongue-like projections and columns of tumor cells, while the Malpighian bodies were reduced to small proportions, being compressed between masses of tumor growth.

The walls of the arterioles showed a marked thickening; the capsule and trabeculæ were moderately thickened, while the sinuses were distended and rigid and small deposits of yellowish brown pigment were present in all parts of the organ.

Bones and Marrow.—At autopsy, the bones of the legs were rather fragile, while the ribs, the sternum, and the bones of the skull were decidedly softened. The medullary cavities of the femur, tibiofibula, humerus, ulna, and radius of both sides were widened and contained masses of tumor growth which in some instances almost filled the cavity. In other places, the growth appeared as small, clearly defined nodules. The bodies of the lower cervical and lumbar vertebræ were also involved. In the lumbar region, the growth projected into the neural canal and pressed upon the spinal cord. Other bones showed no macroscopic evidence of tumor growth and a complete histological examination was not made. Metastases were found, however, in the bones of the pelvis and were doubtless present elsewhere.

The bone metastases were extremely soft and flaky and were composed almost entirely of cells of the vacuolated type with little or no stroma and a very scant blood supply.

Microscopically, the bones showed a distinct widening of medullary spaces with a corresponding reduction in the thickness of the bone. The marrow was in a state of unusually active hyperplasia which was peculiar in that there were very few megacaryocytes and those present showed pycnotic and irregular nuclei with a scant and extremely ragged cytoplasm. Other cells, including both the myelocytic and erythroblastic series, were greatly increased, as indicated in Fig. 24 (middle of femur).

Lymph Nodes.—With the exception of the mesenteric and retroperitoneal groups, the lymph nodes were small, pale, and opaque and intensely indurated. The mesenteric nodes were also small but were deeply pigmented, while those extending from the pelvic region to the celiac axis were slightly swollen, translucent, and moist. Several of these nodes contained soft, opalescent nodules of tumor growth and microscopic examination revealed the presence of many others.

The structure of the metastases in the retroperitoneal lymph nodes differed somewhat from that in the inguinal nodes. There were many focal accumulations of tumor cells lying free in the peripheral and medullary sinuses or imbedded in the substance of the follicles (Fig. 25) which were composed of granular cells with a basophilic cytoplasm and showed numerous mitotic figures. The larger metastases, however, showed an extreme degree of colloid degeneration, and the growth

spread diffusely through all parts of the node with apparently no opposition. The irregularity and indefiniteness of cell outlines, the presence of giant cells not seen in other metastases, and the formation of a peculiar protoplasmic network which characterized the growth in these nodes are illustrated in Fig. 26. Another feature of these lesions was the absence of any stroma except that supplied by the preexisting framework of the node and the presence of few if any newly formed blood vessels. Mitotic figures were fairly numerous but less frequent than in the smaller metastases.

There is some uncertainty as to the origin of the cells composing these lesions. As will be seen later, there was a tumor-like growth in the left testicle of this animal which was composed of very large cells some of which were not unlike the giant cell illustrated in Fig. 26. Hence, the occurrence of cells of this type in this particular location and their absence from other metastases raises the question as to whether these cells were derived from the scrotal or the testicular growth.

Apart from the presence of tumor metastases in isolated groups of nodes, there were decided alterations in all the lymphoid tissues. These were of two kinds. In the first place, there were a more or less complete disappearance of germinal centers and of characteristic germinal cells, or even follicular outlines in many nodes, and a reduction in the number, size, and clearness of definition of the centers in others. Some of these features are illustrated in Fig. 27. At the same time, many nodes showed irregular and, in some instances, rather conspicuous collections of epithelioid (endothelial?) cells throughout the cortex. The sinuses were usually dilated but contained comparatively few cells.

Offsetting these changes, there appeared to be a compensatory increase in the number of nodes, indicated by the frequency with which minute nodes were encountered in sections from various localities; some of these showed a preservation or even a hyperplasia of the germinal centers.

The superficial lymph nodes, as distinguished from those of the abdominal and thoracic regions, showed a distinct fibrous thickening of the capsule and stroma with a thickening of the vessel walls and occasionally a lymphocytic infiltration.

The alterations described in this group of tissues were doubtless due in part to the syphilitic infection but may have been influenced to some extent by the presence of the tumor, since similar changes have been noted in tumor-bearing animals in which no syphilitic infection could be demonstrated.

Pathological Conditions in Organs Not Involved by the Tumor Growth.

While a great deal of valuable information can be acquired from a study of primary and secondary tumor growths and the reactions of surrounding tissues, it is doubtless recognized that fully as much may be gained from a careful survey of other conditions associated with the origin and progress of such a growth. The animal which formed the basis of this investigation presented a number of abnormali-

ties some of which appear to be significant with reference to the two major diseases—syphilis and tumor—and while at present we do not intend to discuss this phase of the subject in detail, it is necessary that the conditions referred to be described for the sake of completeness as well as for future reference.

Clinically, a pronounced scleroderma antedated the recognition of the growth in the scrotum, and post mortem, a number of other conditions were found; these included degenerative changes in the cardiovascular system, chronic inflammatory and degenerative processes associated with atypical epithelial proliferations in the tongue and buccal mucosa, esophagus, bronchi, and left testicle, fibrotic changes in the skin and subcutaneous tissues, and alterations in the endocrine glands.

Cardiovascular System.—The heart was normal, except for occasional foci of chronic myocarditis, the significance of which is uncertain, and a moderate degree of myocardial degeneration. The arterial system, on the other hand, was markedly affected. Many of the smaller arteries were diffusely thickened, while the thoracic aorta contained a series of small saccular dilatations. In these areas, the thickness of the vessel wall was diminished. Viewed from the inner side, there were numerous opaque white or yellowish white lines and dots distributed chiefly over the dorsal surface. There were no nodular thickenings or areas of calcification, however, such as are commonly seen in the aorta of the rabbit.

Histological examination showed the presence of extensive degenerative changes in the media of the aorta and many of the smaller vessels. The appearance presented was that shown in Fig. 28, taken from an artery in the region of the celiac axis. There was no inflammatory reaction associated with these lesions. The process began with a swelling and disintegration of the muscle and connective tissue elements, the elastic fibers showing less alteration. During the earlier stages, cells and intercellular substance gave a distinct basophilic staining reaction and the appearance suggested a mucoid degeneration, but as necrosis developed, the reaction changed, the entire area staining deeply with eosin.

The affected parts of the vessel wall were at first thickened (Fig. 28), but with extension of the necrosis, there was a tendency to a reduction in thickness as may be seen in Fig. 29, taken from the outer edge of an aneurysmal dilatation in the aorta.

A third type of vascular lesion, consisting of a fibrous thickening of the outer coats together with some narrowing of the lumen, has already been referred to in connection with the lymph nodes (see Fig. 27). The nutrient vessels in all parts of the body exhibited changes of this type but they were most pronounced in such tissues as the lymph nodes and the skin.

Skin and Subcutaneous Tissues.—At autopsy, the skin over the ventral surface of the body cut with great resistance. In some places, it was freely movable but was everywhere firmly united to the deeper tissues by coarse and extremely tough strands of fibrous tissue. The subcutaneous tissues were unusually dry. The skin itself was diffusely thickened, the thickening being most marked over the trunk and about the head. Measurements made with calipers after removal of the hair showed a thickness of from 2 to 6 mm. The greatest thickness was in the region of the right shoulder and involved an area approximately 10 cm. square. The measurement at the center was 6 mm., which diminished to 4 mm. at the periphery. The skin over other parts of the thorax varied between 3 and 4 mm. in thickness, along the midline of the back, 2 to 3 mm., and 2 mm. over the abdominal wall. The measurements for these regions are nearly double those of a normal animal of the same type and age.

In addition to the diffuse thickening, there were a number of small shotty nodules in the deeper layers of the skin, chiefly on the back. These varied from minute points to nodules measuring 2 to 3 mm. in diameter.

The thickest portions of the skin were of a leather-like texture, the corium being composed of coarse bundles of pale straw-colored tissue of a fibrous nature. On cross-section, the circumscribed nodules closely resembled the cut end of a nerve.

Thus far, it has been impossible to obtain satisfactory sections of these lesions for microscopic examination, except from the outer edges. Sections from both the diffuse and circumscribed lesions showed obvious abnormalities in the development and distribution of hair follicles as well as the structure of the corium. These conditions may be illustrated by Figs. 30 and 31, which were taken from the outer edge of a small nodule in the skin of the right flank. The structure of this nodule and its relations to other parts of the skin are shown in Fig. 30. It will be seen that the nodule is made up of a collection of large and irregular hair follicles surrounded on all sides by a thick and compact zone of connective tissue forming an elliptical mass which is not clearly demarcated at its periphery. The matrix of the nodule was composed of extremely coarse and angular bundles of hyaline connective tissue as shown in Fig. 31, which may be compared with other figures taken at the same magnification, such as Figs. 11 and 12. There were very few blood or lymph vessels in this tissue, and the walls of all vessels were thickened with a consequent narrowing of the lumen. Cellular elements were also scarce and were confined to a narrow zone immediately surrounding the vessels. The elastic tissues have not as yet been studied on account of technical difficulties.

The hair follicles were abnormal in size, location, arrangement, and structure. Individual follicles varied from minute or atrophic structures on the one hand to irregular giant follicles with radially arranged cells and cortical differentiation on the other, or to follicles of a large or small size composed entirely of undifferentiated germinal epithelium (bulb cells) showing numerous mitotic figures. Several of these structures are shown as dark bodies at the center and toward the extreme right of Fig. 30.

Individual follicles as well as the nests were encircled by connective tissue which was more nearly normal in appearance than that of the body of the nodule, but even here there was some tendency to degeneration and small foci of lymphocytic infiltration were occasionally seen.

The follicles in the papillomatous patches on the lips, which were referred to in the clinical history, presented a similar series of changes. In this instance, however, the tendency was almost entirely toward the production of giant follicles with cortical differentiation analogous to those of the pilot hairs or bristles about the face.

It will be seen that the peculiarities in the growth and arrangement of the hair follicles in the lesions described were not unlike those which existed in the skin surrounding the first and second tumor recurrences or even about the primary tumor itself, and they suggest the existence of a rather widespread abnormality affecting the growth and differentiation of cells forming this group of structures. This possibility is strengthened by the occurrence of an analogous group of changes in the epithelium of the tongue and buccal mucosa, the esophagus, and the testicle, which have not yet been described. However, it should be borne in mind that all these conditions were associated with equally marked alterations in the supporting connective tissue.

Tongue, Buccal Mucosa, and Esophagus.—The lesions present in these organs may be considered together since they represent processes of much the same character and significance. The sides and dorsum of the tongue (Fig. 32, *a*, to be compared with *b* which is normal) showed a series of irregular depressed areas which were smooth and rather translucent in comparison with intervening portions of the mucous membrane which appeared to be thickened and of an opaque white color, especially toward the base and sides of the tongue. Similar markings were found on the mucous membranes of the cheeks.

Microscopically, the depressed areas showed degeneration, necrosis, and exfoliation of surface epithelium associated with a chronic inflammatory reaction in the tissues beneath (Figs. 33 and 34). These changes resulted in destruction of papillæ with consequent smoothing of the surface and a reduction in the thickness of the epithelial covering. There was, however, an active proliferation of epithelial cells in all parts of the mucous membrane, and in the more opaque areas, there was a marked increase in thickness instead of a reduction, with irregular downgrowth of epithelium, as shown in Fig. 35.

The buccal mucosa and the mucous membrane of the upper part of the esophagus contained lesions analogous to those of the tongue. An example of the esophageal condition is given in Fig. 36.

The lesions included in this group are of double interest. The condition of the tongue is analogous in some respects to smooth atrophy and in others to leucoplakia of human syphilis, and is the only instance on record in which such lesions have been observed in an

animal infected with *Treponema pallidum*. The second feature of interest is the existence of chronic inflammatory processes associated with atypical epithelial proliferations in much the same manner as at the point of origin of the original tumor.

Testicles.—The testicles again presented a combination of two conditions. Both testicles were extremely small and on microscopic examination showed atrophy of the seminiferous tubules, slight interstitial fibrosis with considerable thickening of the tunics together with foci of round cell infiltration, chiefly about the vessels of the tunics. In the outer side of the left testicle, there was a mass the size of a small bean which was separated from the remainder of the organ by a thin wall of fibrous connective tissue. It was composed of imperfectly outlined tubular or alveolar structures surrounded by a well developed stroma of fibroblastic cells (Fig. 37). The alveoli were much smaller than the seminiferous tubules and contained large, irregularly shaped cells with vesicular nuclei and a granular cytoplasm which varied in staining reaction from a pale pink to a purplish blue color. The cells were larger than those of the seminiferous tubules but bore no resemblance to the cells of the scrotal tumor. In some places, they preserved the tubular arrangement, while in others, they formed compact and rather irregular alveoli, and occasionally one or more cells appeared to lie free in clefts of the stroma as at the point indicated by the arrow in Fig. 37. The cells were everywhere in a state of active mitotic division but nowhere was there a suggestion of spermatogenesis.

This lesion, if not an independent neoplastic growth, is at least a striking example of a tendency to atypical cell proliferation. The list of tissues showing increased or atypical proliferation of epithelial cells with inflammatory or degenerative changes in the supporting connective tissue, might be farther extended, but the instances cited are sufficient to emphasize this feature of the pathological status of the animal under consideration.

Endocrine Glands.—The thyroid and suprarenals showed decided abnormalities, while changes of a less definite character were present in the thymus and pituitary body.

The thymic mass was of approximately normal size but was composed chiefly of fat with very little thymic tissue.

The thyroid was quite small and of a pale grayish pink color. Microscopically, the outstanding features were irregularity in the size and shape of acini, relatively scant, pale staining, and vacuolated colloid, and an increase in the interstitial tissue. Some of the acini presented an essentially normal appearance, others were small and contained little or no colloid, while the acini of a third group were large and irregularly shaped and the cells lining these acini were large and cuboidal or columnar with irregular nuclei and a decidedly granular cytoplasm; they were frequently several layers deep or formed so called epithelial buds. The colloid in this group of acini stained a pale pink and was extremely vacuolated or even granular in places.

The stroma of the gland was increased and there were a few small areas of lymphocytic infiltration. Some of the blood vessels were slightly thickened, but on the whole they showed no decided abnormality.

The suprarenals, as in the case of the thyroid, were extremely small for an animal of this age and were of a deep orange-yellow color. The medullary area was relatively large, while the cortical zone was comparatively narrow. Microscopically, the cortical cells showed extensive degeneration and necrosis with numerous mitotic figures in the zona glomerulosa.

The changes in the pituitary were less definite. The anterior lobes were unusually prominent, and, microscopically, the cells composing this part of the gland were practically all of one type, their protoplasm containing fine dust-like granules which stained a pale lilac-pink. Other parts of the gland presented an essentially normal appearance.

DISCUSSION.

It would be impossible to undertake a comprehensive analysis of the conditions described in the preceding pages without the introduction of experimental data, which is not advisable at this time. Hence, discussion will be limited to such subjects as can be dealt with upon the basis of material contained in this paper.

It may appear that the conditions which have been described form a rather heterogeneous collection of pathological processes which for the most part bear no relation to one another, but a more careful analysis will show that they are separable into three fairly distinct but closely related groups of phenomena. The first group includes a growth which originated in the scar of a chancre following a mild but persistent syphilitic infection; the second consists of a series of chronic inflammatory conditions associated with active and atypical proliferation of epithelial cells; while the third group of conditions includes a variety of retrogressive processes of widespread distribution, some of which antedated the appearance of the growth in the scrotum while others were of later development and in some instances appear to have been terminal events in the course of the disease. It is apparent that in making this classification there is an unavoidable overlapping of the several groups in that conditions assigned to one group also enter as a part of the processes included in the others.

Clinically, the growth in the scrotum was indistinguishable from a cutaneous syphilide, but its history and the results of pathological examinations have removed all doubt as to its neoplastic nature.

There may be some question, however, as to the proper classification of the tumor. The metastatic lesions in particular present a picture which is usually associated with sarcomata or at least with tumors of mesodermal origin, but a careful examination of a large series of sections of the primary and recurrent growths with surrounding skin areas has led to the conclusion that the tumor is of epithelial origin. It is obvious, however, that the growth does not conform to either the squamous or basal cell type of epithelioma commonly seen in man, although it might be included in the latter group.

The nearest analogy in cell type among normal epidermal structures is found in the cells composing the hair follicle, especially those of the bulb and outer root sheath, and it appears that the cells forming the tumor have followed this line of differentiation. The peculiarities of the tumor might be accounted for, therefore, upon the basis of developmental tendencies of epithelial cells. Thus, in man, the dominant tendency is apparently in the direction of the development of a protective covering of epithelium, the production of hair being subsidiary, whereas in the rabbit, conditions are reversed; the epidermis in general is less highly developed and the protective function is subserved to a greater extent by the growth of a heavy covering of hair. In this animal, as has been pointed out, there were obvious developmental abnormalities of the hair follicles in many parts of the skin which may in itself be of some significance.

No explanation of the origin of the tumor will be offered at this time, but there are certain facts bearing upon the subjects of etiology and malignancy which may be reviewed briefly in order to make clear the nature of the problem presented.

The history of the growth stamps it as a typical instance of the development of a malignant tumor upon the basis of a chronic inflammatory lesion in which a syphilitic infection may have played the part of a predisposing or of an exciting factor. It is apparent that the growth began as a process of atypical epithelial proliferation such as is common to chronic inflammatory lesions of the skin and probably differed in no essential respect from the proliferative changes present in neighboring parts of the skin or from those which subsequently developed in the mucous membranes of the mouth and esophagus.

The time at which the transition to an independent neoplastic growth occurred is not clearly indicated. From the history and from the composition of the lesion first removed from the scrotum, it is certain, however, that this change took place gradually and that there were periods of ineffectual activity before the growth finally became established and assumed the characteristics of a malignant tumor. Even then the progress of the growth was not uninterrupted, but was marked by periods of quiescence or temporary regression such as are frequently observed in other tumors.

It should be borne in mind, however, that these features of tumor growth are not peculiar to tumors but that they represent changes of a type well recognized in lesions or diseases which pursue a relapsing course, and are identical in character with the course of events ordinarily observed in syphilitic infections. Moreover, this feature of the growth was no more than a continuation of the phenomena of relapse which had been taking place in the lesion from which the tumor originated, and in other lesions in this animal. It is apparent, therefore, that the conditions responsible for these changes were not necessarily associated with the tumor itself and that they were not of a purely local character.

A second feature of this case which is deserving of consideration concerns the occurrence of fibroid lesions in the dermis which were noted several months prior to the recognition of the growth in the scrotum. At first, there was only a moderate diffuse thickening of the skin, but eventually, the condition assumed marked proportions with nodular and diffuse lesions. The association of three distinct conditions such as scleroderma, leucoplakia buccalis, and a malignant neoplasm in one individual is most unusual, but the tissue changes in these processes do not differ materially from those frequently observed in cases of malignant disease. As has been pointed out, the lesions of the buccal mucosa and esophagus were of much the same order as that from which the tumor arose, and the fibroid changes in the skin will be recognized as a process of essentially the same character as that seen in the connective tissue of so called scirrhous tumors. In fact, the focal lesions were composed of atypical epithelial growths in the form of hair follicles with a matrix of hypertrophied and hyaline connective tissue, and similar, although less marked changes were

present in the connective tissue surrounding the primary growth and many of the earlier metastatic lesions.

The development of the diffuse fibrous thickening in the skin was undoubtedly influenced by frequent handling over a period of years, but this merely served to accentuate the condition and thus brought out a latent weakness in this group of tissues which otherwise might have escaped notice. The change was progressive, however, and increased after the development of the tumor. In like manner, the growth of connective tissue, which had been feeble at all times, became less and less active, until at the time of death there was little or no connective tissue reaction about metastatic lesions and there was complete failure of union in the operative wounds. Finally, chronic fibrous thickening and acute degenerative changes were found in the walls of the blood vessels, which suggests a course of events not unlike those seen in the skin and in the connective tissue about tumor growths.

Retrogressive changes were not confined to this group of tissues, however, but were present in the parenchymatous organs as well and were especially evident in the lymphoid tissues, the thymus, the thyroid, and the suprarenals. It is thus apparent that there was widespread deterioration of organs and of tissues, much of which antedated the development of the tumor; hence, the tumor did not arise in an organism which was otherwise normal but in one with obvious organic impairment which increased with the progress of the growth.

Moreover, neoplastic changes in the epithelium were not confined to a single focus or even to a small area of the skin but spread in a gradually widening circle and with increasing rapidity, until a considerable part of the skin over the lower abdominal region exhibited changes which differed from those of the original growth chiefly in that they were no longer focal but diffuse in character. At the same time, atypical epithelial proliferations were taking place in other parts of the body and at one point (the left testicle) a growth developed which might be regarded as a second epithelial tumor.

This statement of the circumstances which attended the development and growth of the tumor under consideration will serve to indicate the complexity of the problems of etiology and malignancy

and the necessity for taking into account conditions other than those of a purely local character. In fact, there is every indication that the factor of foremost importance in determining the course of events in this instance was constitutional rather than local. Further than this we shall not go until the results of our experimental work have been reported.

SUMMARY AND CONCLUSIONS.

A malignant tumor which developed at the site of a primary syphilitic lesion in the scrotum of a rabbit, about 4 years after inoculation, and eventually led to the death of the animal, has formed the basis for an extensive series of investigations beginning with the study of the spontaneous tumor. The present report contains the clinical history of the animal from the time of inoculation to its death and the results of pathological examinations.

The most important events recorded may be summarized as follows:

1. A mild but persistent syphilitic infection with an occasional relapse and the eventual development of chronic inflammatory lesions in the skin associated with atypical epithelial proliferations.

2. The occurrence, at about the same time, of extensive degenerative changes in the dermis accompanied by an atypical growth of hair follicles, with the production of diffuse and nodular areas of thickening and induration (scleroderma).

3. The development of a growth in the skin of the left scrotum which recurred after removal and spread diffusely over adjacent parts of the skin, and metastasized to the regional lymph nodes and to internal organs.

4. The transplantation of the growth to other rabbits by intratesticular inoculation and the successful propagation of the growth over a period of nearly 2 years (twenty generations).

5. The development of a cachexia and of pressure phenomena from metastases involving the cervical and lumbar regions of the spinal column which eventually led to the death of the animal.

6. The discovery, post mortem, of an extensive leucoplakia of the tongue and buccal mucosa, chronic inflammatory lesions in the esophagus with atypical epithelial proliferation, and a nodular growth in the left testicle differing in character from that in the scrotum.

7. The presence of extensive degenerative changes in the vascular system, degeneration of the parenchymatous organs, atrophy of the thymus and lymphoid system, with chronic lymphadenitis, atrophy, degeneration, and necrosis of the suprarenals, and atrophy and hyperplasia of the thyroid with chronic thyroiditis.

The conclusion was reached that the growth in the scrotum represented a neoplasm of epithelial origin which was composed of cells allied to those found in the bulb and root sheath of the hair. No explanation was offered for the origin or unusual malignancy displayed by the tumor pending the introduction of experimental data. It was stated, however, that the evidence presented was sufficient to warrant the assumption that the factor of foremost importance in the development of the tumor was constitutional rather than local.

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EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which have not been retouched. Gross objects are represented at their natural size. The photomicrographs were taken from sections stained with hematoxylin and eosin or with methylene blue and eosin (Figs. 16 and 17).

PLATE 31.

FIG. 1. Chronic inflammatory reaction in the left scrotum 3 years and 7 months after inoculation.

FIG. 2. Primary growth in the left scrotum 4 years and 4 months after inoculation.

FIG. 3. Recurrence of the tumor growth in the left scrotum with metastases in the inguinal nodes 3 months after excision of the primary growth. The scar from this operation is seen below the recurrent growth.

FIG. 4. Cross-section of the primary tumor with part of the surrounding skin area. The points marked on this figure refer in some instances to exact areas described or illustrated in succeeding photographs or to general localities in instances in which the photographs were taken from other sections of the same growth. See the text for descriptions. × 12.

FIG. 5. Epithelioid type of growth from the oldest of the three nodules composing the primary tumor mass (*a*, Fig. 4). × 450.

PLATE 32.

FIG. 6. Primary growth. Changes in the epidermis and papillary layer of the skin in the region of *b*, Fig. 4. × 200.

FIG. 7. Primary growth. Epidermis and papillary layer of the skin showing intracystic growths of epithelium. The same general area as Fig. 6. × 200.

FIG. 8. Primary growth. Cells from the interior of nodule *B* (*c*, Fig. 4). × 900.

FIG. 9. Primary growth. Arrangement of cells and structure of growth in the same area as Fig. 8. × 450.

FIG. 10. Part of the primary growth from area *e*, Fig. 4. This section shows the origin of the tumor cells from the epidermis with a gradual transition in the appearance of the cells as the growth extends downward and becomes continuous with nodule *B*. × 200.

FIG. 11. Primary growth. Tumor cell emboli in the lymphatics of the skin outside the area of tumor growth (*f*, Fig. 4). × 200.

PLATE 33.

FIG. 12. Part of an accessory growth in the skin above the primary nodule (*g*, Fig. 4). Note the general hyperplasia of the epithelium, the arborization of the epithelial process, the transition to basal cells, the perilymphatic arrangement of the basal cells, the sharp delimitation of the growth, and the absence of keratinization and of cellular reaction. × 200.

FIG. 13. Part of an accessory growth from the same area as Fig. 12. The section was taken from the periphery of an atypical hair follicle and the growth simulates this structure. × 200.

FIG. 14. Atypical epithelial proliferation with chronic inflammatory reaction in the skin at the outer edge of the tissue removed with the primary growth. This is of interest in connection with the recurrent growth. × 200.

PLATE 34.

FIG. 15. Diffuse epithelial growth from the skin at the outer edges of the first cutaneous recurrence. Note the similarity between the growth of epithelium in this section and that in Figs. 6, 10, and 12. × 200.

FIG. 16. Atypical growth of hair follicles in the skin about the first cutaneous recurrence. × 200.

FIG. 17. Metastasis, left inguinal node. The section shows the general character and distribution of cells forming these lesions, areas of necrosis, fibrous thickening of the capsule and vessels, and invasion of tumor cells. × 200.

PLATE 35.

FIG. 18. Convex surface and cross-sections of the liver showing the appearance of the metastatic nodules.

FIG. 19. Liver. Part of an old metastatic nodule surrounded by a thick wall of dense fibrous tissue which is being invaded by the tumor cells. Note the type of tumor cell composing this growth as contrasted with those in Figs. 20 and 21. × 450.

FIG. 20. Liver. A more recent lesion showing colloid or mucoid degeneration of tumor cells with slight reaction at the outer margins. × 450.

PLATE 36.

FIG. 21. Liver. A third type of growth which combines some of the features of the two preceding lesions but is more malignant in character than either of these. × 450.

FIG. 22. Lung. Emboli of tumor cells in pulmonary vessels. × 450.

FIG. 23. Kidney. Tumor cell emboli in glomerular capillaries. Note also the hyaline thickening of the capillary walls and the degeneration of the tubular epithelium. × 450.

PLATE 37.

FIG. 24. Femur. Hyperplasia of the bone marrow. × 450.

FIG. 25. Retroperitoneal lymph node from the celiac region. Mass of tumor cells in a peripheral sinus with beginning growth in the substance of the node. × 200.

FIG. 26. Retroperitoneal lymph node from the lower lumbar region. A diffuse tumor growth of the colloid type containing mononuclear giant cells which resemble those of the growth in the left testicle. Compare with the cells of the scrotal tumor in Fig. 8, taken at the same magnification, and with the testicular growth in Fig. 37, which is half this magnification. × 900.

FIG. 27. Superficial cervical lymph node. Note especially the absence of germinal centers and the thickening of the capsule, and the walls of vessels in the node and surrounding tissues, indicated by arrows. × 50.

PLATE 38.

FIG. 28. Artery from the region of the celiac axis showing degeneration and necrosis in the media. Note the increased thickness in these areas. × 50.

FIG. 29. Thoracic aorta. Longitudinal section at the outer edge of an area of dilatation. The thickness of the wall diminishes at this point and there is almost complete necrosis of the muscle and connective tissue elements. The elastic fibers are fairly well preserved. × 100.

FIG. 30. Section of the outer edge of an indurated cutaneous nodule from the ventral surface of the right flank. The drawn lines indicate the general outlines of the nodule which is composed of nests of atypical hair follicles surrounded by

coarse bundles of hyaline connective tissue, the individual fibers of which are greatly enlarged. × 12.

FIG. 31. Hypertrophy and hyaline degeneration of connective tissue from the center of Fig. 30. Compare with Figs. 11, 12, and 14 at the same magnification and note the absence of capillaries and connective tissue cells. × 200.

PLATE 39.

FIG. 32, *a* and *b*. Upper surface of the tongue from specimens preserved in Kaiserling's solution. (*a*) Syphilitic-tumor rabbit. (*b*) Normal rabbit. The distortion in *a* is largely artificial and accidental. The irregular markings indicate the important surface abnormalities.

FIG. 33. Cross-section of tongue, *a* in Fig. 32, showing necrosis and erosion of the outer layers of epithelium in a translucent area. There is a moderate chronic inflammatory reaction in the mucosa with perivascular infiltration and slight hyperplasia of the epithelial cells. × 100.

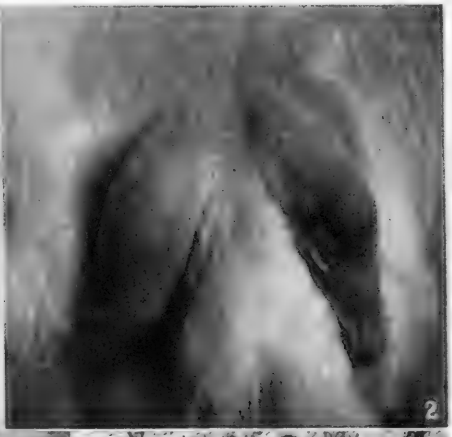
FIG. 34. A more advanced stage of the same process that is shown in Fig. 33. Papillæ are entirely absent from this area and the epithelium is deeply eroded, while the inflammatory reaction and epithelial hyperplasia are increased. × 100.

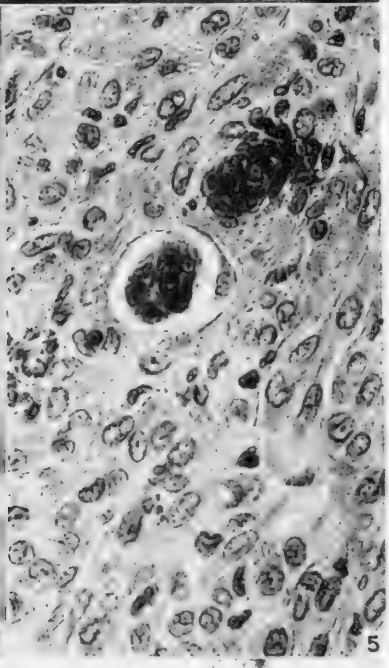
FIG. 35. Tongue. Atypical hyperplasia of epithelium from one of the more opaque white areas toward the sides of the tongue (*a*, Fig. 32). A leucoplakia-like lesion. × 100.

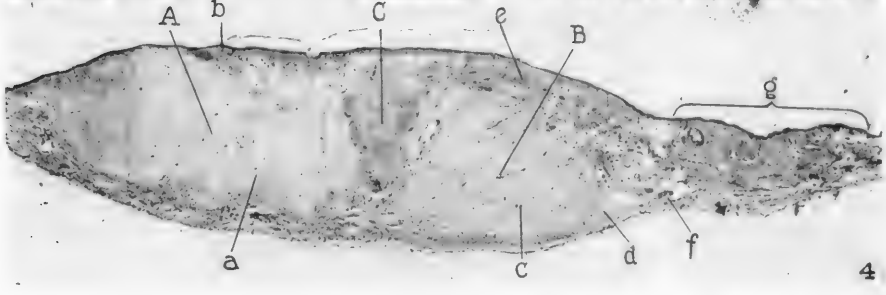
PLATE 40.

FIG. 36. Esophagus at the level of the larynx. Atypical hyperplasia of the epithelium with areas of degeneration and necrosis associated with a slight inflammatory reaction. There are numerous mitotic figures in the lower layers of the surface epithelium and in the gland-like structures of the deeper tissues. × 200.

FIG. 37. Section of the growth in the left testicle. The tubular structures composing this lesion were from one-quarter to one-half the size of the atrophied seminiferous tubules, while the cells averaged two to three times the size of those in the tubules. The outlines of the tubular structures are lost in places and some of the cells are free in the fibroblastic stroma. Compare these cells with those of the metastatic growth in Fig. 26. There are numerous mitotic figures in all parts of the section. × 450.







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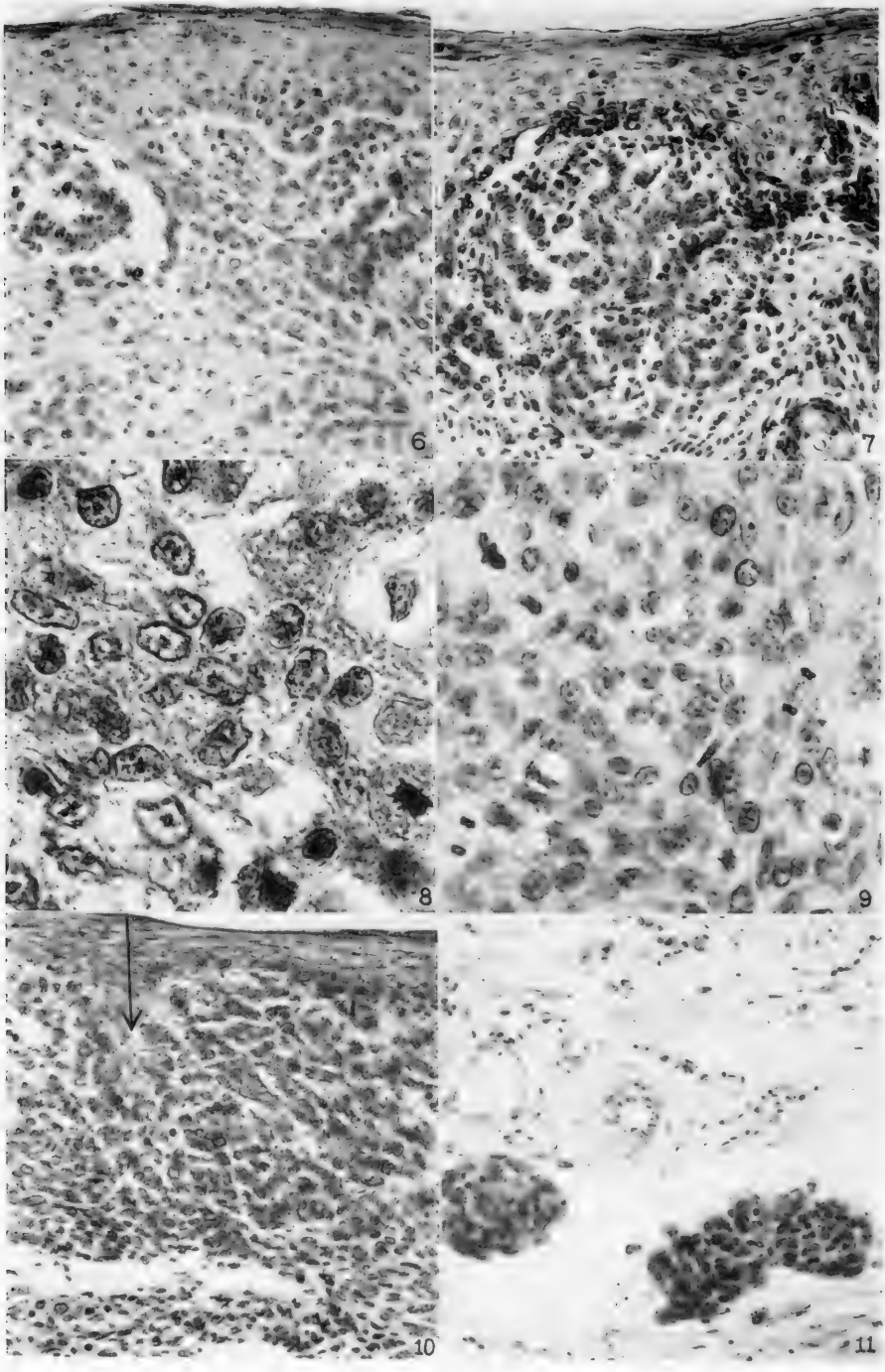

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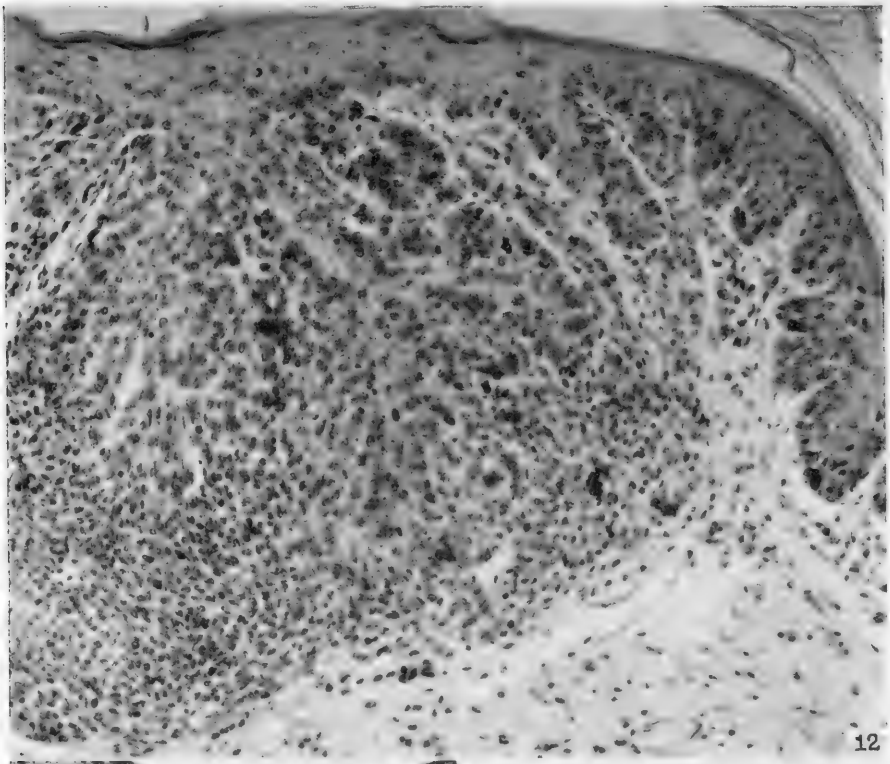

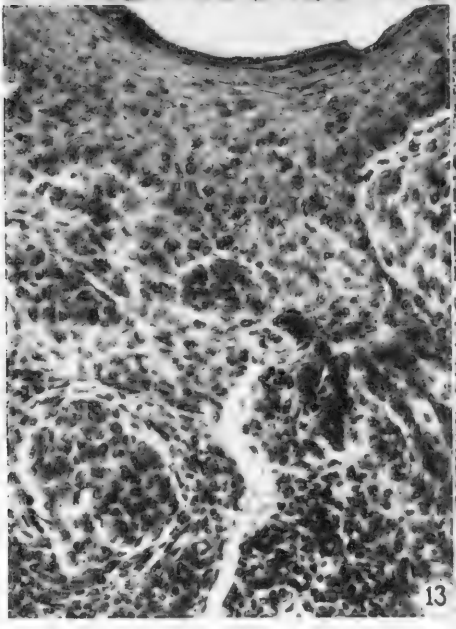

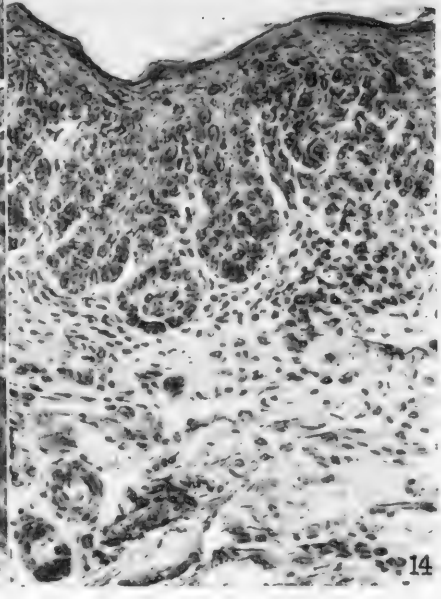

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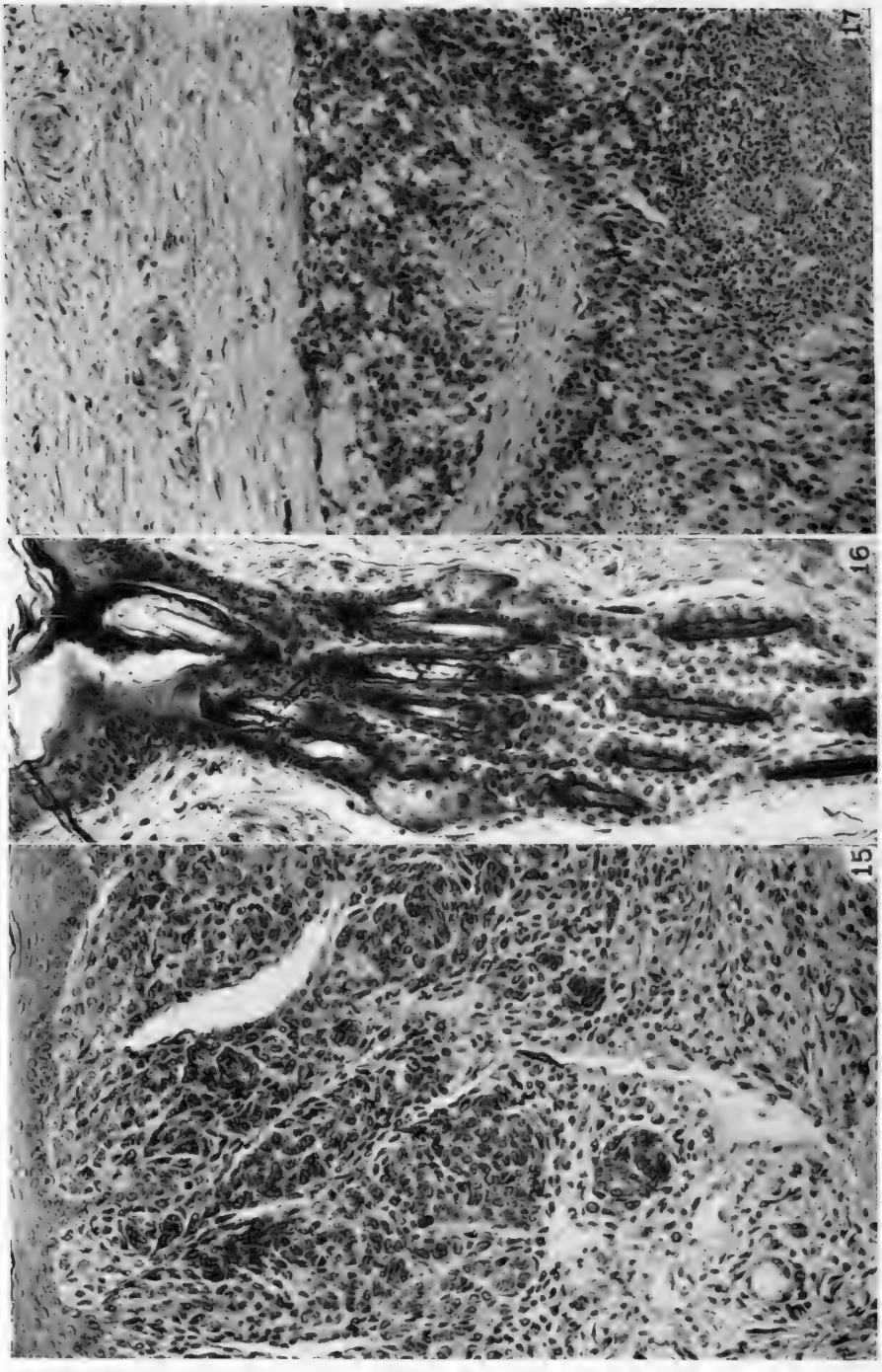

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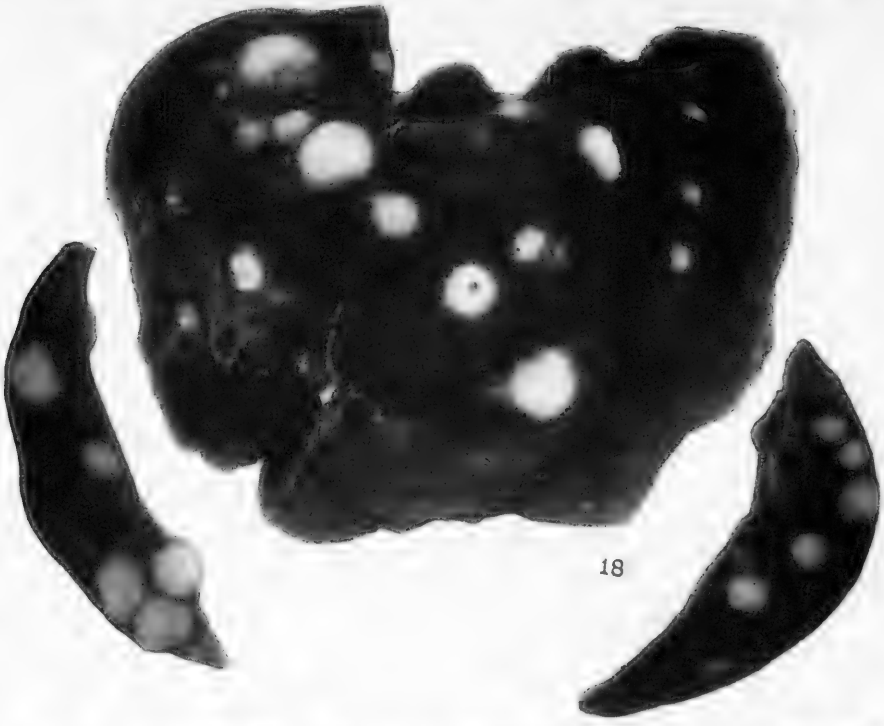

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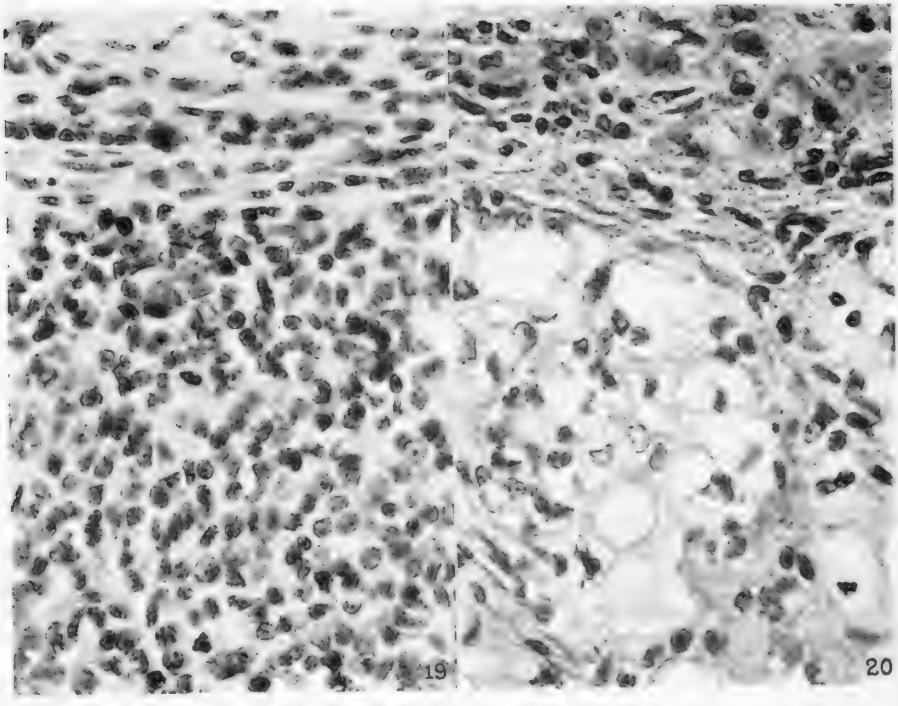

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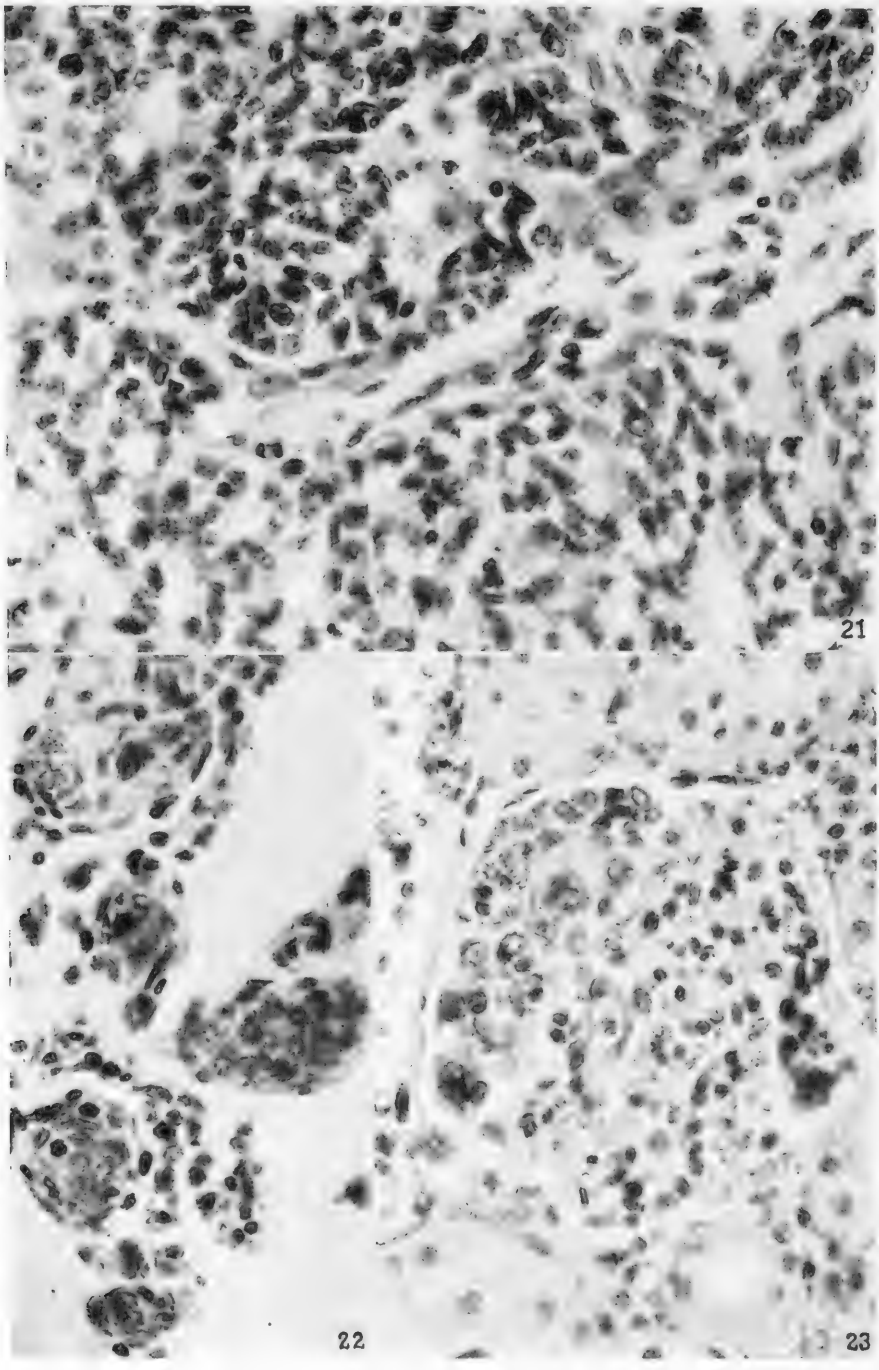
(Brown and Pearce: Malignant tumor of the rabbit. I.)



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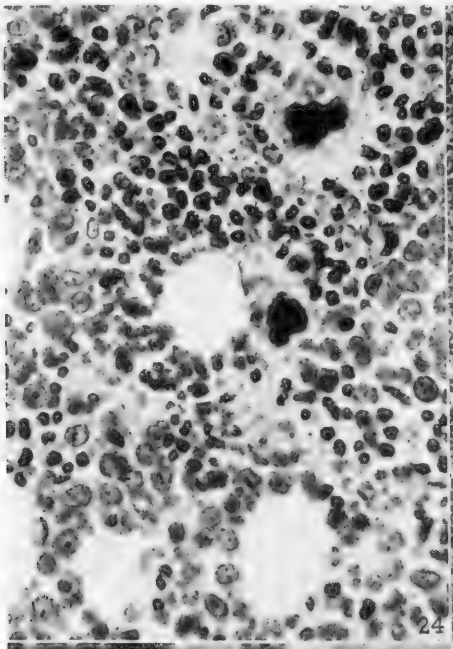
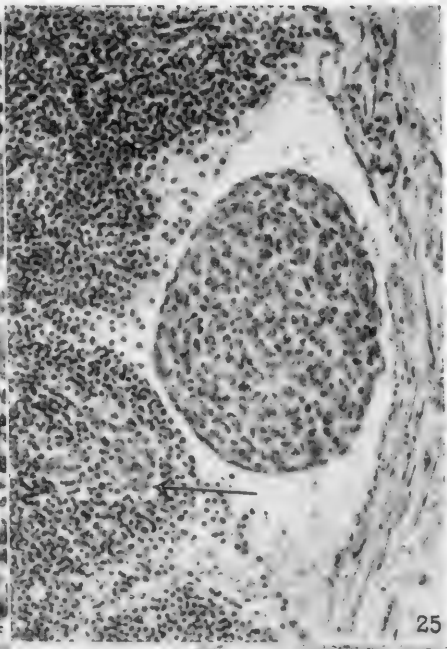
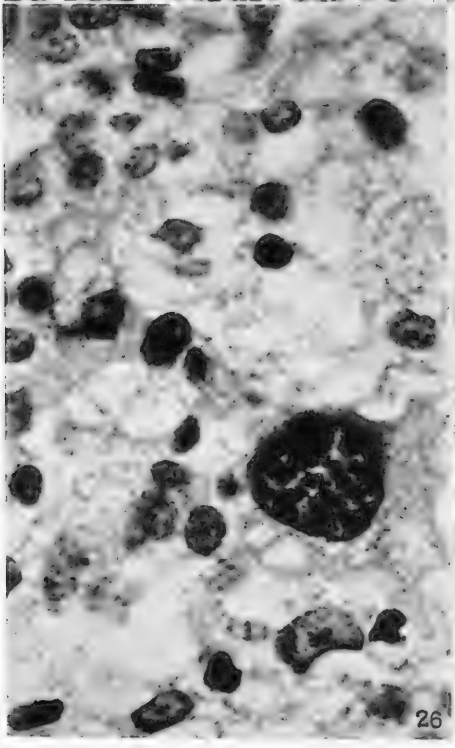
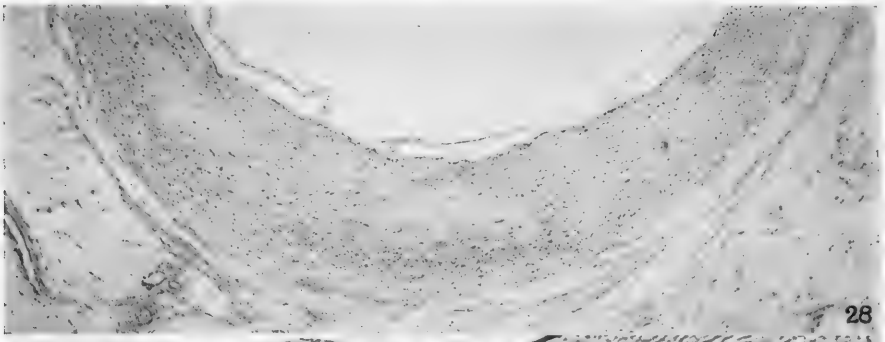






(Brown and Pearce: Malignant tumor of the rabbit. I.)



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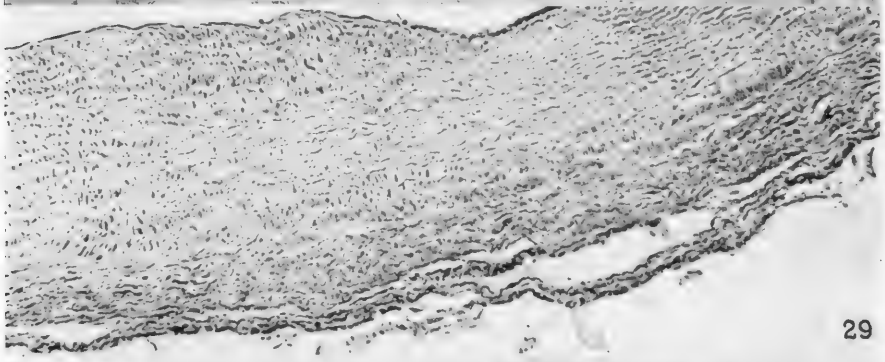

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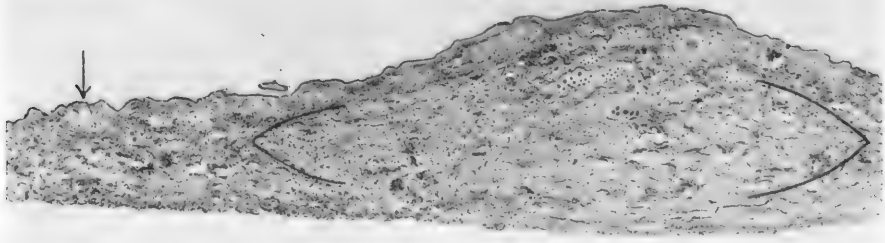

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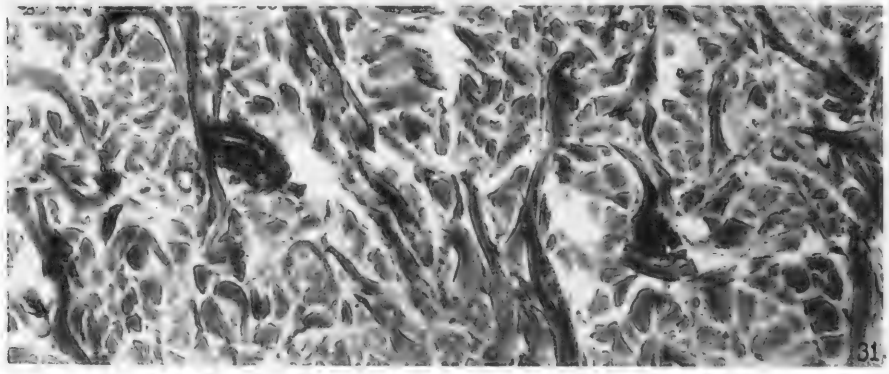

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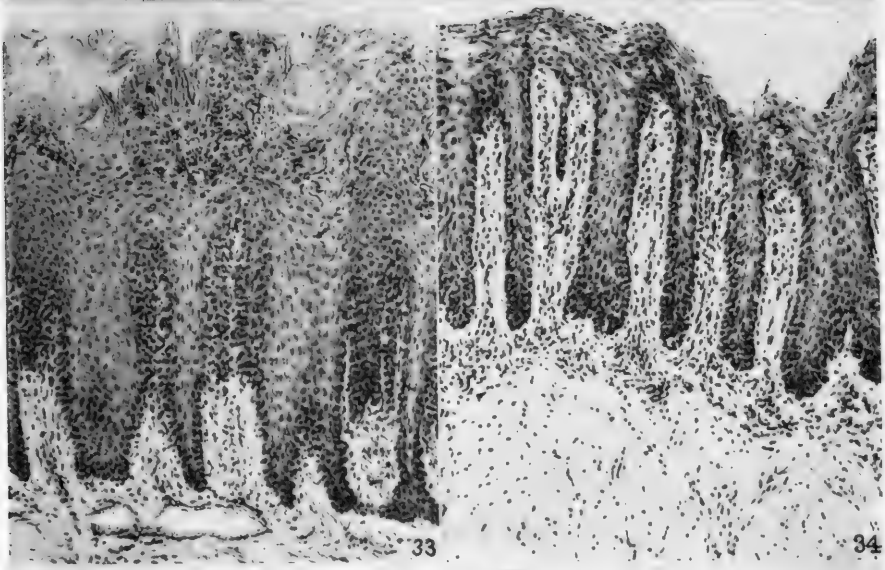
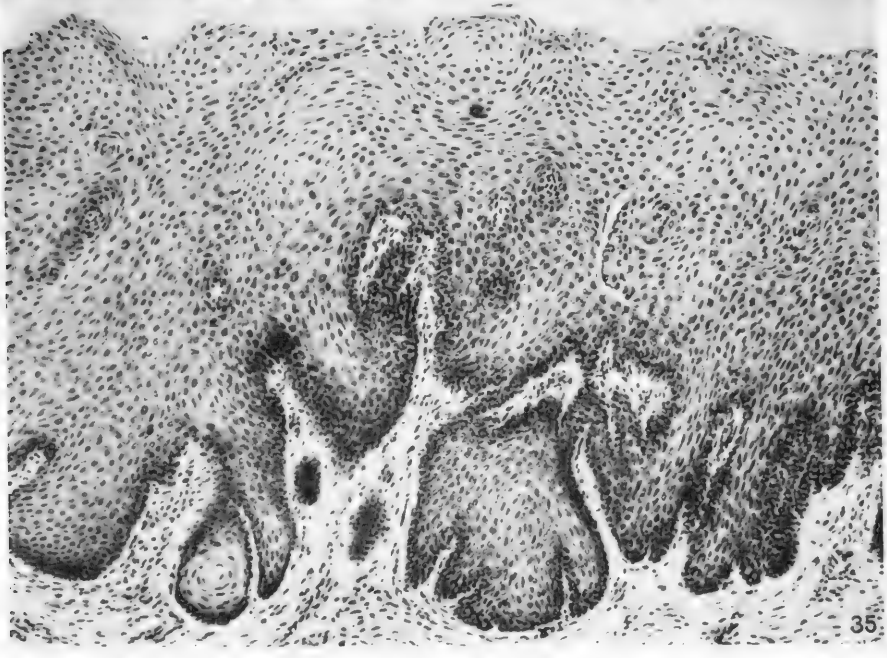
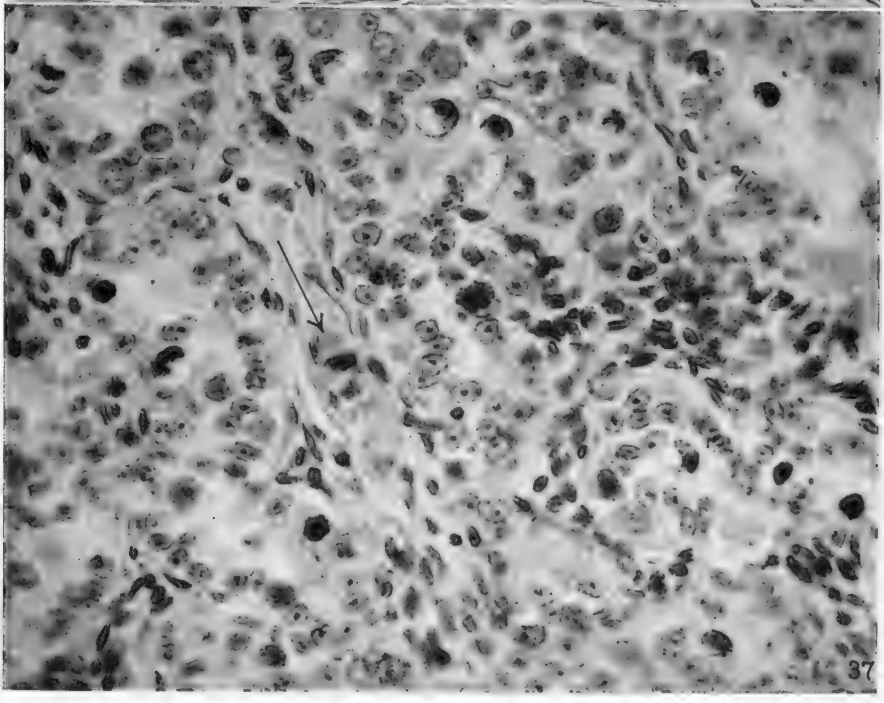
(Brown and Pearce: Malignant tumor of the rabbit. I.)





(Brown and Pearce: Malignant tumor of the rabbit. I.)



(Brown and Pearce: Malignant tumor of the rabbit. I.)

STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

II. Primary Transplantation and Elimination of a Coexisting Syphilitic Infection.

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Plates 41 to 43.

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The report (1) dealing with the spontaneous tumor upon which this series of investigations is based, contained a brief reference to attempts which were made to transfer the tumor to other rabbits, indicating that these attempts had proved successful and that the tumor had been propagated for a number of generations. In view of the difficulties which others have experienced in the transplantation of tumors of the rabbit and as a means of conveying a general conception of the behavior of the tumor when transplantation was first accomplished, a more detailed account of these phases of our work will be given in the present paper.

Primary Transplantation.

An early diagnosis of a malignant growth simplified the problem of primary transplantation to the extent of permitting several attempts to be made before the animal succumbed. There was a complicating factor, however, in the coexistence of a syphilitic infection, the significance of which was not known at that time. It was not known whether the tumor was inseparably bound up with a local infection or to what extent the systemic infection might have influenced its development. At any rate, there were two possibilities to be considered; first, the transmission of syphilis by the inoculation of normal animals, and, second, the influence which an established syphilitic infection might exert upon the growth of the transplanted tumor.

First Series.—While a tentative diagnosis of a malignant tumor was made from the nodule excised in October, 1920, it was several months before the growth recurred and developed sufficiently to provide material for transplantation. The first attempt to transplant the tumor was made on January 27, 1921. The material used came from a metastatic growth in a lymph node of the left inguinal region. Inoculations were made according to the conventional method of subcutaneous implantation of small pieces of tumor tissue. A transplant was made in the right groin of the original animal, and six male rabbits, three normal and three syphilitic, were inoculated in the scrotum; one female was inoculated at the base of the right ear.

The homograft grew fairly actively and was subsequently used in the inoculation of other animals. The transplants in the normal animals and one of the syphilitics with a comparatively recent and still active infection appeared to grow for upwards of 2 to 3 weeks, but the nodules remained small and indurated and eventually regressed, so that for practical purposes, growth results in these animals may be regarded as negative. They were valuable, however, in that they served as a warning against placing too much reliance upon subcutaneous transplantation and in this way aided in the orientation of the next series of inoculations.

Second Series.—The second attempt to transplant the tumor was made on March 3, 1921. In this instance, material was taken from several different sources, including a portion of the cutaneous recurrence, the homograft in the right groin, and parts of metastases in both the right and the left groins. This series contained fourteen male rabbits, ten normal and four syphilitic, all of which were inoculated subcutaneously, either in the groins or scrotum, with small bits of tissue as in the preceding experiment. In addition, however, three of the normal rabbits were inoculated in the right testicle with 0.3 cc. of a cell emulsion from the transplant of the original animal.

The results of the subcutaneous inoculations were much the same as before. Of the ten normal animals, two died soon after inoculation, five showed a suggestion of growth, in two there was slight but definite growth, while the tenth animal was definitely negative. Of the four syphilitic animals, one showed a definite growth, and while the nodule remained small, it persisted for at least 2 months before final regres-

sion set in. It may be noted that this animal was one that had exhibited an unusually severe syphilitic infection as regards both the extent and the duration of the lesions.

The results of the testicular inoculations were somewhat better. Small but transient tumor nodules developed in two of the three animals, while in the third, the growth measured approximately 1 cm. in diameter at the end of a month and was still increasing actively when it was removed to supply material for another series of inoculations and was the source from which the existing line of stock transfers was started (used for second generation).

From this experiment, it was also found that the transmission of syphilis had to be reckoned with. Of the eight normal rabbits that survived, five developed definite syphilitic lesions at the site of inoculation and it appeared not unlikely that the infection was more general.

Third Series.—Soon after the second series of transplants was made, the condition of the original animal became such that it had to be killed and a third series of rabbits was inoculated as a safeguard against the loss of the tumor. By this time, we were convinced that little was to be hoped for from subcutaneous inoculations. It had been found that the pieces of tissue introduced beneath the skin stimulated a vigorous granulomatous reaction much the same as any foreign body and that the initial efforts at growth were almost immediately suppressed by the formation of a dense fibrous capsule, the tumor cells being incarcerated and smothered out, as it were. For the same reasons, it appeared that the opportunities for growth might be improved by the introduction of the cells into some organ in which connective tissue was less abundant or in which nutritive conditions were better than those afforded by the subcutaneous tissues. The indications were that the testicles might meet these requirements; hence, the third series of inoculations was carried out by this route. The material used was taken from metastatic nodules in the liver. The tissue was finely minced and carefully rubbed up in a mortar, an emulsion being prepared by the addition of a small amount of normal salt solution. 0.5 cc. of this emulsion was injected into each testicle of four normal rabbits by means of a syringe and an 18 gauge needle.

Three of these animals developed tumors which grew slowly at first and then more actively and eventually metastasized to various

parts of the body. One animal in particular showed extensive tumor growths in the abdominal viscera with metastases in the ciliary body of one eye and in the upper jaw.

None of the animals in this group developed syphilitic lesions, and while there is no assurance that they were not infected, the contrast in this respect with animals inoculated with material from lymph node metastases is worthy of note, especially in view of the more vigorous growth of the tumor in the absence of outspoken lesions of syphilis.

Solution of the Problem of Transplantation and Elimination of the Syphilitic Infection.

The success obtained by the use of intratesticular inoculations gave promise of a satisfactory solution of the problem of transplantation, but the advantages of this method as a means of propagating the tumor and the behavior of the transplanted growth were still to be determined upon a larger series of animals; there was also the question of a syphilitic contamination to be disposed of, and these problems were taken up with the second generation of transfers.

Second Generation.—The inoculations of the second generation were made on April 7, 1921, from the testicular growth of the animal previously indicated in Series 2. In this instance, the tissue was finely minced with scissors so that, when salt solution was added, it could be drawn into a syringe through an 18 gauge needle which was used for making the inoculations. The animals inoculated included four syphilitics, seven rabbits previously inoculated with tumor tissue, and twenty-two normal animals, only the last of which need be considered at this time. The normal rabbits were divided into three groups: ten of them were inoculated in the right testicle, nine were given an intracutaneous inoculation on the ventral surface of the sheath, and three were inoculated in both the testicle and the sheath.

The results of this series of inoculations were very striking and may be described in greater detail than the preceding experiments as a means of illustrating the important part which intratesticular inoculation has played in the transplantation of the tumor and the behavior of the growth at the beginning of transplantation. For convenience of analysis, the essential data of this experiment are assembled in Table I.

By reference to Table I, it will be seen that only one animal of the nine inoculated on the sheath (Group A) developed a tumor of any considerable size. The growth in this instance was essentially the same as that illustrated in Figs. 1 to 4. The primary tumor grew actively for a period of approximately 8 weeks, during which time a small metastatic nodule developed in the right inguinal lymph node. Regression then took place with apparently complete recovery, and at autopsy, there was no evidence of healed metastases in other parts of the body.

A second animal (No. 2) developed a small nodule at the site of inoculation which appeared to be something more than the usual granulomatous reaction but was not sufficiently definite to warrant a positive diagnosis of tumor growth. As will be seen, three animals of this group were kept under observation for 6 to 7 months; the others were reinoculated in the right testicle at the end of 7 weeks with a rather anomalous result in that all but one of them developed testicular tumors from the second inoculation.

This fact is mentioned because a growth subsequently appeared in one of these animals (No. 3) at the site of the original inoculation. The growth did not appear until long after the second inoculation, but there is no way of determining whether it represented a delayed development from the first inoculation or a metastasis from the testicular tumor, the location of which might have been determined by the previous trauma. At any rate, the lesion on the sheath was still active when the animal died $4\frac{1}{2}$ months after the second inoculation. Death was due to the development of widespread and extensive metastases which were accompanied by gradual emaciation, weakness, and anemia, the loss in weight amounting to 550 gm., or more than 25 per cent of the normal body weight. The extent of the growth and the emaciation at the time of death are illustrated in Fig. 5.

The part played by the sheath lesion in the production of this condition is uncertain, but it appears that metastases such as those in the inguinal lymph nodes (Fig. 5) may have originated from this source. In all probability, however, this animal illustrates a condition of affairs essentially the same as that of the second group of animals (Group B) in which there was a simultaneous inoculation of testicle and sheath. This opinion is based upon the fact that in no

TABLE I.

Results of Tumor Inoculations in the Second Generation.

Animal No.	Result of inoculation.	Incubation period.	Extent of primary growth.	Duration of growth.	Length of observation.	Presence of metastases.	Disposition of animal.	Remarks.
			Group A. Intracutaneous inoculation.					
		days		*wks.*	*wks.*			
1	+	9	+++	8	29	+	Killed.	Spontaneous recovery.
2	±	?	?	?	7	−	Reinoculated.	Reinoculation +.
3	−?	−	−	−	7	−?	"	See text for results on this animal.
4	−	−	−	−	24	−	Killed.	No tumor growth.
5	−	−	−	−	25	−	"	" " "
6	−	−	−	−	7	−	Reinoculated.	Reinoculation +.
7	−	−	−	−	7	−	"	" +.
8	−	−	−	−	7	−	"	" +.
9	−	−	−	−	7	−	"	" −.
			Group B. Intracutaneous and intratesticular inoculation.					
1	+ +	9 9	++++ +++	10 10	10	+++	Killed.	Encapsulated tumor in right testicle.
2	+ +	10 6	+++ ++	29 5	29	−	"	" " " " "
3	+ ±	9 ?	+++ ?	24	24	−	"	

Group C. Intratesticular inoculation.

1	+	14	++++	4	4	−	Killed.	Growth still active when killed.
2	+	9	++++	4	9	−	"	Castrated 4 wks. after inoculation.
3	+	9	++++	9	9	++	"	" 4 " " "
4	+	9	++++	8	8	+++	"	" 4 " " "
5	+	6	++++	7	7	++	"	Growth still active when killed.
6	+	6	++++	10	10	+++	"	" " " " "
7	+	9	++++	24	24	+++	"	Castrated 7 wks. after inoculation.
8	+	9	++++	11	11	+++	Died.	Intercurrent infection.
9	+	9	++++	12	24	−	Killed.	Spontaneous recovery.
10	+	9	++++	29	29	++++	"	Extremely weak when killed.

? indicates result uncertain; −, negative; ±, suggestive positive; +, positive, slight primary growth or metastases; ++, moderate primary growth or metastases; +++, large primary growth or numerous metastases; and ++++, extremely large primary growth or extensive metastases.

In Group B, the double columns of figures refer to testicle and sheath respectively.

other instance has an intracutaneous inoculation led to the death of the animal.

Of the three animals in the second group (Group B, Table I), two developed large tumors at both sites of inoculation, while in the third, there was a good growth in the testicle but the result of the sheath inoculation was uncertain.

The first animal of the group may be taken as an example of the height of activity attained by the tumor at this time and under these conditions. The growth of the primary tumors from the 5th to the 10th week is shown in Figs. 1 to 4. It will be seen that when the animal was killed at the end of the 10th week, there were well developed metastases in the inguinal lymph nodes of both sides. The animal was in good physical condition. The weight had fluctuated between 1,825 and 2,025 gm., the initial weight being 1,850 and the final weight 2,000 gm., giving a net gain of 150 gm. At autopsy, metastases were found in the inguinal and flank nodes, the omentum, the mesentery, the perirenal fat, the kidneys, and the wall of the right ventricle.

The course of the tumor growth in the two other animals of this group was somewhat different. In one animal, both tumors grew actively for a period of about 5 weeks, at which time the lesion on the sheath began to regress and completely disappeared. The testicular growth persisted and at the time the animal was killed (29 weeks after inoculation) formed a mass about 5 to 6 by 2.5 to 3 cm. in diameter which was necrotic at the center and enclosed in a thick fibrous capsule. The animal was in good physical condition and no active or healed metastases were found.

The third animal showed no definite growth on the sheath but the course of the testicular tumor and the autopsy findings were much the same as those of the second.

The ten animals of Group C need not be considered individually, except in a few instances. By reference to Table I, it will be seen that all the animals inoculated in the testicles developed large primary tumors with an incubation period of from 6 to 14 days. These animals were used in various ways for further transplantation experiments and for studying the progress of the tumor growth. Three (Nos. 1, 5, and 6) were killed for pathological examination 4, 7, and

10 weeks respectively after inoculation; three animals (Nos. 2, 3, and 4) were castrated at the end of 4 weeks and later killed (8 to 9 weeks) and one other animal (No. 7) was castrated after 7 weeks. This left three rabbits in which there had been no operative interference with the progress of the tumor and one of these (No. 8) died from an intercurrent infection (11 weeks). The two remaining animals (Nos. 9 and 10) together with the one castrated at 7 weeks (No. 7) were held under observation for 24 to 29 weeks before being killed for final examination.

The first animal killed (4 weeks) showed no recognizable metastases, but two of the three animals castrated at the same time showed recurrence of the tumor in the scrotum and cord with metastases to internal organs when killed 4 and 5 weeks later (Nos. 3 and 4). At the end of 7 weeks (No. 5), the growth was found to have extended to the abdominal cavity with metastases to numerous organs, and a similar but more marked picture was presented by the animal killed after 10 weeks (No. 6). Metastases were also present in the animal that died from an intercurrent infection (11 weeks).

The picture presented by all these animals was much the same in character. There was an active growth of the primary tumor which, if undisturbed, filled the entire testicle and usually extended up the cord in the form of isolated nodules situated in the cord itself or in the serous membranes of the inguinal canal and adjacent parts of the abdominal cavity. In addition, metastases were present in the mesentery and omentum, in the retroperitoneal tissues, and in various internal organs. The appearance presented by the primary tumor and the extension of the growth along the inguinal canal may be illustrated by Figs. 6 and 7, taken from Animal 5, killed 7 weeks after inoculation. The histology of the early growth is shown in Fig. 8, which represents a section from the testicle of Animal 3, 4 weeks after inoculation.

Only three animals of this group were held beyond the 11th week. One of these (No. 9) showed an apparently complete recovery, and when killed (24 weeks), there was no evidence of metastases. The second animal (No. 7) had been castrated, but local recurrence had taken place, followed by encapsulation and partial regression. At autopsy, however, large metastatic growths were found in the pelvis, the mediastinum, and the right suprarenal.

The other animal of the group showed a slowly progressive tumor growth with palpable metastases in the abdominal organs and gradual development of cachexia. When the animal was killed after 29 weeks, he had become extremely emaciated and weak. At autopsy, extensive metastases were found in the abdominal and thoracic organs, the condition presented being much the same as that shown in Fig. 5, with an especially marked involvement of the kidneys, one of which is shown in Fig. 9.

The results obtained in this series of animals will serve to indicate the general characteristics of the growth and the potentialities of malignancy as they appeared at this time under conditions of transplantation which were favorable to the growth of the tumor. These results also convinced us of the superiority of intratesticular inoculation over subcutaneous or intracutaneous inoculations as a means of propagating the tumor, and this type of inoculation has since been employed as the routine method for stock transplantations. The only respect in which the method used in this experiment has been changed is in the substitution of a homogeneous cell emulsion, prepared by mincing and grinding the tumor tissue, for the more tedious process of preparing a finely minced suspension of tumor fragments.

Elimination of Syphilitic Infection.—None of the animals of the second tumor generation showed outspoken lesions of syphilis, but since the infection was known to be widely disseminated among those of the first generation, steps were taken to remove the possibility of transmitting this infection through tumor inoculations. For this purpose, several animals with actively growing tumors were subjected to vigorous treatment with arsphenamine. The treatment given to different animals varied from a single intravenous injection of 20 mg. of arsphenamine per kilo of body weight to two or three doses of 15 mg. per kilo given at weekly intervals. This gradation in treatment was adopted as a precaution against any harmful effects which the drug might exert upon the tumor itself and to secure at the same time the necessary degree of treatment. Among the animals treated, there were some known to be infected with *Treponema pallidum*, and the ultimate effects upon the transmissibility of the infection were controlled by subsequent inoculation of normal animals with emulsions prepared from the popliteal nodes of the treated animals.

The treatment with arsphenamine produced no harmful effects upon the tumor growth which were immediately demonstrable. On the contrary, the tumors in these animals grew much more rapidly than those of untreated animals of the same series. Eventually, however, there was a suggestion that spontaneous recovery might have been facilitated by this treatment, but the number of animals was too small and the period of observation too short to permit of any definite conclusion upon this point, and as yet the experiment has not been repeated.

Test inoculations were not made from the lymph nodes of treated animals for several months after treatment in order to allow sufficient time for any surviving organisms to recover. The results of these inoculations were negative in all cases. This in itself furnishes no assurance that the infection had been completely eradicated, but as a further provision of control, the test animals were reinoculated with *Treponema pallidum* at the end of 3 months and developed syphilitic lesions in the testicles.

While awaiting the outcome of tests as to the efficacy of the treatment which had been given, transfers were made from an animal that had received two doses of 15 mg. of arsphenamine per kilo of body weight. This line has been perpetuated as our stock strain and no further evidence of syphilitic infection has been seen in animals inoculated with material derived from this source.

Method of Transplantation.—As a result of the experiments reported, intratesticular inoculation was adopted as the routine method of transplantation, and the details of the method now used differ very little from those described above. Care is exercised in the selection of a tumor which is actively progressing, and, as a rule, transplantations are made at intervals of from 2 to 4 weeks, depending upon the rate of growth and the general behavior of the tumors which are to be used for inoculation.

The animal is anesthetized and castrated with aseptic precautions. The tumor is then sectioned and the most actively growing parts are placed in a dish or mortar and finely minced with scissors. Occasionally a suspension of this material is made by the addition of 0.85 per cent sodium chloride solution, but, as a rule, the minced tissue is rubbed up in a mortar and a homogeneous cell emulsion is prepared.

In either case, the inoculation is made by the injection of 0.1 to 0.3 cc. of this material with a syringe fitted with an 18 or 20 gauge needle.

The preparation of material is carried out aseptically but no antiseptic precautions are used in making inoculations other than to sponge off the surface of the scrotum with a 50 per cent solution of alcohol in some instances. For routine purposes, animals are inoculated in only one testicle. The dose used in different series of animals is largely a matter of judgment as to the quality of the material and the thickness of the suspension. A cell emulsion is used in preference to a suspension of tissue fragments purely as a matter of convenience, as thus far no material difference in results has been observed. The method as described is extremely simple and has given an almost uniform series of takes with a high percentage of malignant tumor growths.

DISCUSSION.

The ease with which this tumor was transplanted and has been maintained for more than twenty generations will doubtless be apparent. Success is attributed in part to good fortune and in part to the recognition of the true significance and importance of simple pathological processes as factors in the growth of the transplanted tumor. We were fortunate in discovering the tumor before the condition of the animal became critical and more fortunate in being able to recognize the cause for the failure of subcutaneous transplantations and to devise methods which obviated these difficulties. The chief element of success in the transplantation of the tumor was the interpretation of the reaction in the skin and subcutaneous tissues as an ordinary process of repair or of encapsulation which was capable in itself of determining the fate of the tumor cells, and the correctness of this interpretation was amply sustained by the success which followed the use of methods based upon this assumption.

It is also of interest to note that while the tumor was first transplanted from a graft in the original animal, transplantation was accomplished with equal facility from metastases in the liver, and the growth in these animals was more malignant than that in animals inoculated from the graft.

In regard to the syphilitic infection which was transmitted to some of the earlier animals, attention may be called to the fact that so far as is known, these infections all originated from inoculations made with material from lymph nodes; that is, no instance of outspoken syphilitic infection developed among the animals which were inoculated only from the skin, the graft, or liver metastases. A further feature of interest is the fact that in no instance was there a simultaneous activity of both processes. There were only a few animals in which a tumor growth was obtained, but none of them developed syphilitic lesions, and with one exception, the tumor failed to grow in animals with an established syphilitic infection or in those in which syphilitic lesions developed following tumor inoculation.

The percentage of active tumor growths obtained by testicular inoculation and the malignancy displayed by the tumor in the first and second generations are somewhat unusual, and while these conditions were due in part to the method of inoculation, they are also indicative of a high degree of proliferative activity or resistance on the part of the tumor cells. No accurate forecast of the course of the tumor during the first and second generations can be given, since so few of these animals were held for any considerable period of time. The occurrence of metastases was determined in the great majority of animals, and in several instances, it was shown that they must have occurred as early as the 4th to the 7th week. It seems safe to assume that eventually the majority of the animals with metastases to internal organs would have died as a result of the tumor growth, but probably not all of them.

Some indication of the probable course of the disease at this period in its history may be obtained from a group of two animals of the first generation and five of the second which were held for 6 to 7 months after inoculation. These animals were inoculated in the testicles, but two of them received an intracutaneous inoculation in addition. Two animals virtually succumbed to the tumor growth, being killed when it was apparent that they could survive for only a short time, but both of them lived approximately 7 months after inoculation. There were two other animals in which active metastatic lesions were found at autopsy, and these probably would have succumbed in time. Two animals showed what may be termed a partial recovery or an arrested growth in that encapsulated and partially necrotic primary growths

were still present but no metastases were found. Both of these received a cutaneous as well as a testicular inoculation. Finally, there was one animal in which an apparently complete recovery had taken place.

Upon this basis, one might forecast a prolonged course of disease with a mortality of more than 50 per cent. These figures may be somewhat misleading, however, in that the animals of the second generation were hardly representative of their group in respect to age and activity of growth of the primary tumors, and as we know, these factors are of some importance. Discounting the results upon this basis, it is not unlikely that the mortality would have been increased somewhat and the length of survival materially shortened. Nevertheless, a slowly progressive course appears to have been a characteristic feature of the disease at this time as compared with later generations. It is obvious, however, that the transplanted tumor possessed highly malignant characteristics in even the earliest generations. Since this time, the tumor has exhibited some rather striking variations in its behavior which will be made the subject of a future communication.

SUMMARY.

Several attempts were made to transplant a spontaneous malignant tumor in a syphilitic rabbit before a method was devised which proved successful. Subcutaneous and intracutaneous inoculations were unsuccessful on account of the vigorous granulomatous reaction which was aroused by the introduction of tumor fragments into these tissues. This difficulty was overcome by the use of intratesticular inoculations. With this method, a good growth was obtained in practically all animals. It appeared possible, however, that a syphilitic infection had been transmitted along with the tumor, and hence treatment with arsphenamine was instituted as a means of eliminating this infection. A study of the transplanted tumor in the first and second generations showed that it possessed a high degree of malignancy. Metastases occurred at an early period in the majority of animals, and while some of them appeared to recover, the indications were that more than 50 per cent might be expected to succumb to the effects of the tumor growth within a period of 6 to 7 months.

The method of intratesticular inoculation now in use is described in detail.

BIBLIOGRAPHY.

1. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 601.

EXPLANATION OF PLATES.

The illustrations are all from photographs which have not been retouched.

PLATE 41.

FIGS. 1 to 4. Rabbit 1, Group B. Primary tumors in the right testicle and sheath showing stages in development between the 5th and 10th weeks after inoculation. Fig. 4 shows metastases in the inguinal lymph nodes. × 1.

PLATE 42.

FIG. 5. Rabbit 3, Group A. Primary and metastatic tumor growths with emaciation. × ½.

PLATE 43.

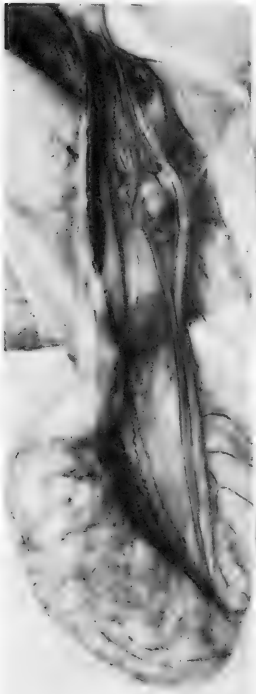
FIGS. 6 and 7. Rabbit 5, Group C. Primary growth in the testicle with metastases along the inguinal canal 7 weeks after inoculation. × 1.

FIG. 8. Microscopic appearance of the transplanted tumor 4 weeks after inoculation. × 150.

FIG. 9. Kidney of Rabbit 10, Group C. × 1.

(Pearce and Brown: Malignant tumor of the rabbit. II.)

(Pearce and Brown: Malignant tumor of the rabbit. II.)



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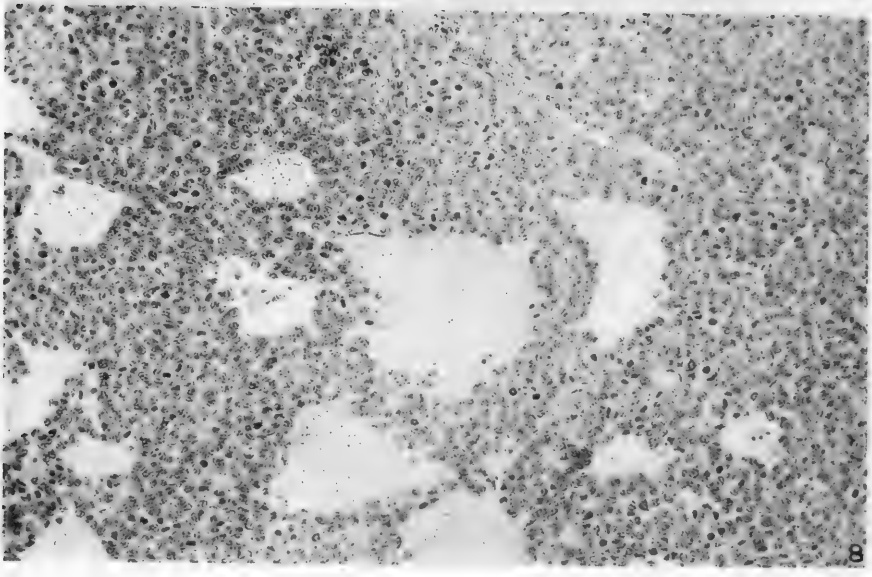

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(Pearce and Brown: Malignant tumor of the rabbit. II.)

STABLE SUSPENSIONS OF AUTOAGGLUTINABLE BACTERIA.

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The study of the factors determining the agglutination of bacteria reported in a previous paper[1] has shown that agglutination occurs whenever the electric potential between the organism and the surrounding fluid falls below a certain critical value. The charge on organisms in general is negative in alkaline, and positive in acid media. Between the two extremes the charge is low and the organisms agglutinate. This is the acid agglutination zone. It was also found that the charge may be increased and the suspension thereby made more stable by (1) the addition of alkali or acid in such a way as to bring the suspension to a pH far from the isoelectric point; (2) the addition of trivalent or tetravalent ions; and (3) the addition of substances which affect the critical potential. The stability is decreased by (1) the addition of alkali or acid so as to approach the isoelectric point of the organism; and (2) the addition of any ion of the opposite charge to that of the organism.

It is possible, from these results, to predict conditions which will make a given suspension either more or less stable at will.

The present paper is an attempt to apply these results to organisms which are normally granular.

Certain organisms, such as *Streptococcus hæmolyticus* and Type G, bacillus of rabbit septicemia, grow in a granular fashion and yield unstable suspensions which make immunological agglutination studies difficult. This obstacle has been overcome with certainty in the case of Microbe G, rabbit septicemia bacillus. It has been shown

[1] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655. De Kruif, P. H., and Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 127, 139.

in a preceding paper[2] that the acid agglutination optimum of Type G lies between pH 5.0 and 3.5. With some strains, agglutination may occur at pH 6.0 or even at pH 6.6. But at pH 7.0 to 7.5 all strains so far studied have proved stable.

This fact was utilized in a preceding study.[3] Suspensions of Type G were made in distilled water, and the serum under test was diluted with buffer pH 7.1. Under such conditions, the controls remain in suspension, as do the mixtures of Type G with quantities of highly dilute immune serum.

TABLE I.

Agglutination of Type G by Normal and Immune Serum at pH 7.5 and 6.6.

Dilution with glycocoll-Naacetate-Na_2HPO_4 buffer M/12.

Serum.	pH	Concentration of serum (43° C. for 14 hrs.)							Control.
		0.1*	0.05	0.025	0.0125	0.006	0.003	0.0015	No serum.
Immune	7.5	C.	C.	C.	C.	++	+	−	−
Normal	7.5	++	++	+	Tr.	−	−	−	−
Immune	6.6	C.	C.	C.	C.	C.	C.	+	+
Normal	6.6	"	++	+	+	+	+	+	+

* In the tables the concentration of serum is given in cubic centimeters of serum per cubic centimeter of solution. To obtain the final dilution of serum and suspension divide by 2.

Table I presents the results of immunological agglutination tests of Microbe G, in which the serum is diluted in buffer pH 7.5 and 6.6. At pH 6.6 some agglutination occurs, even in the control, in which serum is replaced by buffer pH 6.6. This renders the readings unclear and makes difficult the determination of a definite end-point. At pH 7.5, on the contrary, the reaction is clear-cut, no complete agglutination occurring in the controls or in the high dilutions of serum. Buffer pH 7.1 can be used with equal safety.

[2] De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 395.
[3] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxvi, 309.

Preparation of Stable Suspensions of Streptococcus hæmolyticus.

Dochez, Avery, and Lancefield[4] found that many autoagglutinable strains of streptococcus could be stabilized by washing in distilled water and suspending in this medium, but that some races always remained granular and were therefore unsuitable for agglutination test. For the present study five strains of *Streptococcus hæmolyticus* from scarlet fever patients were obtained which showed intensely granular growth.[5]

The strains (Nos. 1, 3, 4, 5, and 6) were seeded into 10 per cent rabbit serum broth. All grew in a sedimentary fashion, leaving the supernatant fluid clear. These sediments from 16 hour growths were washed four times and suspended in distilled water. Flocculation occurred in every instance. Attempts were made to suspend the organism in buffer solutions of high pH concentration, in the hope that an effect similar to that with Type G might be secured. The buffer solution used was the glycocoll-Na acetate-Na_2HPO_4 mixture described in preceding papers.[1] Suspensions were made in buffer mixtures pH 9.0 to 7.0. In the molecular concentration ordinarily employed—M/12—the results at all pH concentrations were disappointing. Flocculation still occurred in the majority of instances.

When the buffer mixtures were diluted with distilled water to M/96 (pH 8.5) better results were obtained, but with two strains rather rapid autoagglutination still occurred.

Suspensions were next prepared in diluted solutions of Na_3PO_4 (M/128). The pH was very high, > 9.0 to phenolphthalein. The suspensions obtained were quite stable, but the agglutination occurring when immune serum was added was slow and indistinct.

Stabilization of Streptococcus hæmolyticus by 0.001 N NaOH.

Sediments from distilled water washings of the five strains were suspended in 0.001 N NaOH. The pH of this solution, after contact with air, is about 7.0. The resulting suspensions were by far the most stable of any obtained. Only with Strain 1 did marked settling occur,

[4] Dochez, A. R., Avery, O. T., and Lancefield, R. C., *J. Exp. Med.*, 1919, xxx, 179.

[5] These strains were obtained through the kindness of Dr. Dochez.

and even here actual granulation did not take place, and the suspension could easily be brought back to uniform turbidity.

The 0.001 N NaOH suspensions were now tested against normal and streptococcus immune rabbit serum with the results recorded in Table II. The sera were diluted with distilled water. Table II shows that a definite difference exists between the normal and immune serum agglutination of Strains 1, 3, 4, and 6. Strain 5 was agglutinated by neither normal nor immune serum. The result still left much to be desired, since the agglutination by immune serum with Strains 3, 4, and 6 was not complete in any dilution; *i.e.*, the suspension was now too stable.

TABLE II.

Agglutination of 0.001 N NaOH Suspensions of Streptococcus hæmolyticus by Normal and Immune Rabbit Serum.

Strain No.	Immune rabbit serum.						Normal rabbit serum.					
	0.1	0.05	0.025	0.0125	0.006	0.003	0.1	0.05	0.025	0.0125	0.006	0.003
1	C.	++	C.	+	Tr.	Tr.	++	Tr.	Tr.	Tr.	Tr.	Tr.
3	++	++	Tr.	−	−	−	Tr.	"	−	−	−	−
4	++	+	+	+	−	−	"	−	−	−	−	−
5	Tr.	−	−	−	−	−	"	−	−	−	−	−
6	+	+	−	−	−	−	"	−	−	−	−	−

The electrolyte concentration of the serum suspension mixtures is extremely low (0.0005 N NaOH). The sensitivity of the suspension to agglutination will therefore be increased by the addition of electrolyte (positive ions) and there should be a zone of electrolyte concentration in which the suspension is stable by itself but will agglutinate in the presence of antibody.

Such suspensions of all five strains were consequently tested against decreasing concentrations of NaCl, and it was found that good stability resulted when M/320 concentrations of NaCl were employed. 0.001 N NaOH suspensions of Strains 3, 4, 5, and 6 remained stable in this NaCl concentration. Strain 1 settled noticeably, but did not granulate.

The following experiment was now made with normal and immune rabbit serum. These sera were diluted 1:10 in M/320 NaCl. The

1:10 dilutions were then carried by 2's in M/320 NaCl to 0.003 of the original serum. 1 cc. of each dilution was then mixed with an equal volume of suspensions of all of the strains in 0.001 N NaOH. The mixtures were incubated at 43°C. for 12 hours, and readings taken. The results, which are recorded in Table III, are strikingly better than those recorded in Table II. The difference in four instances between normal and immune serum is clear-cut and distinct. It would appear that the immune serum possesses little or no antibody for Strain 5.

TABLE III.

Agglutination of 0.001 N NaOH Suspensions of Streptococcus hæmolyticus by Serum Diluted with M/320 NaCl Solution.

Final concentration of NaOH 0.0005 N; final concentration of NaCl M/640.

Strain No.	Immune rabbit serum.						Normal rabbit serum.					
	0.1	0.05	0.025	0.0125	0.006	0.003	0.1	0.05	0.025	0.0125	0.006	0.003
1	C.	C.	C.	C.	C.	++	++	++	+	Tr.	Tr.	Tr.
3	"	"	"	"	+	+	+	Tr.	Tr.	−	−	−
4	"	"	"	"	C.	+	++	+	+	Tr.	Tr.	Tr.
5	++	++	+	+	+	Tr.	+	+	+	+	−	−
6	C.	C.	++	+	+	"	++	++	+	Tr.	Tr.	−

Since the method presented has been subjected to a limited number of tests, it is not claimed as an infallible one for agglutination studies of granular strains of streptococcus. It is presented here in the hope that it may be applied by other investigators in this field, and its true value ascertained by extensive and rigorous trial.

SUMMARY AND CONCLUSIONS.

Autoagglutinating cultures of Type G, bacillus of rabbit septicemia, can be rendered stable by suspension in glycocoll-acetate-phosphate buffer mixtures pH 7.5 and 7.1. Distilled water suspensions can also be employed if the normal and immune serum under test is diluted with buffers of this pH instead of with 0.85 per cent NaCl.

Five intensely autoagglutinable strains of *Streptococcus hæmolyticus* have been stabilized by washing and by suspending in 0.001 N NaOH. Specific agglutination of these stable suspensions by rabbit antistreptococcus serum could be demonstrated by diluting the serum with M/320 NaCl.

ANTAGONISTIC GROWTH-ACTIVATING AND GROWTH-INHIBITING PRINCIPLES IN SERUM.

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It is known that the serum of an adult animal restrains the proliferation *in vitro* of homologous fibroblasts and that its inhibiting power increases progressively as the age of the animal advances.[1] It has also been observed that, when serum has been shaken for several hours,[2] or heated for half an hour at 65°C.,[3] its restraining influence on cell activity is still more marked. By contrast, serum which contains leucocytic secretions[4] or aqueous extracts of certain tissues[5] becomes a better culture medium for homologous cells. These facts led to the supposition that the action of serum on homologous fibroblasts was not due to one inhibiting substance only, but rather to the combined action of two substances, one activating and the other inhibiting. In order to test this hypothesis, an attempt was made to separate an activating substance from serum, and to find out whether, after the removal of this substance, the inhibiting effect of serum on homologous cells was enhanced.

EXPERIMENTAL.

The serum was obtained, as a rule, from plasma of chickens between 1 and 2 years of age, and occasionally from younger or older chickens. 10 cc. of serum were diluted with 90 cc. of distilled water and precipitated by bubbling CO_2 through the solution for 10 minutes. The precipitate was thrown down by centrifugation and, after the

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.
[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 399.
[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.
[4] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.
[5] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

supernatant fluid had been pipetted off, dissolved in 10 cc. of Tyrode solution. The pH of the solution was brought to between 7.6 and 8. The supernatant fluid was evaporated *in vacuo* until its volume was reduced to 10 cc., and the pH was adjusted to that of the precipitate solution.

Tyrode solution containing the dissolved precipitate and the treated serum were compared to Tyrode solution and the original serum respectively. The medium was composed of one volume of plasma and three volumes of solution or serum, and contained 1/60 embryonic tissue juices. The tissues were taken from a 10 year old strain of fibroblasts.[6] The rate of growth was ascertained by the increase in the area of the tissues in 48 hours, and the measurements were made according to a technique previously described.[7] As the differences in the rate of growth were expected to be very small, it was necessary to use the greatest care in the application of the technique in order to prevent the errors from being larger than 7 per cent. To express such differences, we have used the ratio between the relative tissue increase in the cultures containing dissolved precipitate or treated serum and that observed in the appropriate controls.

1. Action of the Substances Precipitated from Serum by CO_2 on Homologous Fibroblasts.—The action on homologous fibroblasts of the substances precipitated by CO_2 could not be directly compared with that of the original serum because the rate of cell proliferation increases when the concentration of serum proteins in the culture medium is diminished. As the precipitate consists of only a part of the serum proteins, a better growth of the fibroblasts in the precipitate solution than in the original serum would not necessarily mean that the precipitate contains growth-activating substances. It could occur merely as an effect of a lesser concentration of an inhibiting substance. Therefore, the action of the precipitate had to be compared to that of Tyrode solution, in which the migration of fibroblasts is known to be always more active than in the serum of an adult animal.

In fourteen groups of experiments, a medium containing 75 per cent of Tyrode solution as such was compared to a medium containing the same amount of the solution of the substances precipitated by

[6] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[7] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

CO_2 (Table I). The average rate of migration was 13 per cent greater in the medium containing the CO_2 precipitate. As the errors due to the technique are about 7 per cent,[7] it would appear that the precipitated substances have a weak activating effect on homologous fibroblasts. As the proliferation of fibroblasts in Tyrode solution is more active than in the plasma of a chicken 1 or 2 years old,[8] the rate of growth in the precipitate solution is much greater than in the original serum.

TABLE I.

Action of the CO_2 Precipitate on the Rate of Growth of Homologous Fibroblasts.

Group No.*	Culture No.	Rate of growth in.		Ratio, $\frac{E}{C}$.	Tyrode solution.		Precipitate solution.	
		Tyrode solution (C).	Precipitate solution (E).		pH	Refractive index.	pH	Refractive index.
1	29987	2.86	3.54	1.23	8.0	1.3280	7.4	1.3314
2	30050	3.47	3.70	1.06	8.0	1.3280	7.6	1.3286
3	30098	4.20	4.43	1.05	8.0	1.3280	7.8	1.3304
4	30154	4.22	4.50	1.07	8.0	1.3280	7.8	1.3304
5	30184	4.17	4.20	1.01	8.0	1.3290	7.6	1.3320
6	30229	3.24	3.72	1.15	8.0	1.3290	7.8	1.3320
7	30282	3.65	4.20	1.15	8.0	1.3290	7.6	1.3316
8	30312	3.15	3.42	1.09	8.0	1.3290	7.6	1.3316
9	30650	3.27	3.60	1.10	8.0	1.3290	7.8	1.3298
10	30709	2.92	3.28	1.12	8.0	1.3290	7.6	1.3305
11	31217	3.80	3.86	1.02	8.0	1.3280	7.8	1.3301
12	31239	3.63	3.81	1.05	8.0	1.3280	7.7	1.3291
13	1928	4.26	5.33	1.25				
14	1959	2.75	5.14	1.87				
Average.....				1.15				

* Each group averaged from four to five experiments.

2. Action of the Treated Serum on Homologous Fibroblasts.—The treated serum was reduced to its original bulk of 10 cc. by evaporation *in vacuo* and compared to the original serum as regards its action on homologous fibroblasts. Since serum is rendered less concentrated by the removal of the globulins, a lessening in its inhibiting action on fibroblasts might be expected. Should it prove instead to be more growth-restraining than the original serum, in spite of its lower

[8] Carrel and Ebeling,[1] Text-fig. 12.

concentration in proteins, there would be no doubt that the removal of the globulins had increased its inhibiting power. In fourteen groups of experiments the rate of proliferation of fibroblasts was found to be 26 per cent slower in the serum deprived of globulinthan in the original serum (Table II).

TABLE II.

Action of the Treated Serum on the Rate of Growth of Homologous Fibroblasts.

Group No.*	Culture No.	Rate of growth in.		Ratio, $\frac{E}{C}$.	Original serum.		Treated serum.	
		Original serum (*C*).	Treated serum (*E*).		pH	Refractive index.	pH	Refractive index.
1	31187	2.38	1.69	0.71	7.8	1.3356	7.6	1.3310
2	31191	3.68	2.98	0.81	7.8	1.3356	7.6	1.3310
3	31192	5.36	3.46	0.64	7.5	1.3360	7.6	1.3310
4	31210	2.91	2.17	0.75	7.5	1.3360	7.6	1.3310
5	31209	2.64	2.06	0.78	7.4	1.3320	7.4	1.3310
6	31201	3.99	3.21	0.80	7.4	1.3320	7.4	1.3310
7	31218	3.26	2.87	0.88	8.0	1.3385	8.0	1.3345
8	31225	4.45	3.45	0.78	8.0	1.3385	8.0	1.3345
9	31228	3.25	2.84	0.87	8.0	1.3385	8.0	1.3345
10	31234	2.87	2.19	0.77	7.8	1.3370	8.0	1.3320
11	31240	2.94	2.36	0.80	7.8	1.3370	8.0	1.3320
12	1774	5.40	3.82	0.70				
13	1784	6.10	3.02	0.50				
14	1786	5.30	2.36	0.45				
Average.....				0.74				

* Each group averaged from four to five experiments.

3. Action on Homologous Fibroblasts of Normal Serum, and of Serum Diluted and Reduced by Evaporation to Its Original Volume.—The question arose as to whether the increase in the inhibiting action of serum might not be due merely to some deterioration of the proteins occurring during evaporation. To determine the point, 10 cc. of serum were diluted with 90 cc. of distilled water and reduced by evaporation to the normal volume. The actions of the normal and of the evaporated sera on homologous fibroblasts were compared (Table III). No marked differences were found in the rate of growth.

4. Simultaneous Action of Treated Serum and Globulin Precipitate on Homologous Fibroblasts.—The precipitate was dissolved in the

serum from which it came and the action of the solution on fibroblasts was compared to that of the original serum (Table IV). The differences in the rate of growth of fibroblasts cultivated in normal

TABLE III.

Action of Diluted and Reconcentrated Serum on the Rate of Growth of Homologous Fibroblasts.

Experiment No.	Culture No.	Rate of growth in.		Ratio, $\frac{E}{C}$.	Original serum.		Diluted and reconcentrated serum.	
		Original serum (*C*).	Diluted and reconcentrated serum (*E*).		pH	Refractive index.	pH	Refractive index.
1	31249	2.39	2.39	1.00	8.2	1.3373	8.2	1.3369
2		2.93	2.70	0.92				
3		3.25	3.25	1.00				
4		2.91	2.85	0.98				
5		3.64	3.42	0.94				
Average				0.97				

TABLE IV.

Action of the CO_2 Precipitate Dissolved in the Treated Serum on the Rate of Growth of Homologous Fibroblasts.

Experiment No.	Culture No.	Rate of growth in.		Ratio, $\frac{E}{C}$.	Original serum.		CO_2 precipitate dissolved in treated serum.	
		Original serum (*C*).	CO_2 precipitate dissolved in treated serum (*E*).		pH	Refractive index.	pH	Refractive index.
1	31271	3.60	3.20	0.89	8.1	1.3372	8.0	1.3365
2		3.50	3.30	0.94				
3		2.84	2.72	0.96				
4		3.17	2.90	0.91				
Average				0.93				

serum, and in the solution of the precipitate in the supernatant serum were only 7 per cent; that is to say, they came within the limits of error of the method.

CONCLUSIONS.

The substances precipitated by CO_2 from the serum of chickens 1 or 2 years old slightly increased the activity of pure cultures of a 10 year old strain of fibroblasts, an indication that a growth-promoting substance had been obtained from the serum. The rate of growth of fibroblasts was slower in serum deprived of its globulins and the increase of the inhibiting effect of the treated serum is thus manifested. It would appear that the inhibiting action of serum is referable to one or several growth-activating substances which precipitate by CO_2, and to one or several growth-retarding substances which remain in the supernatant serum after the precipitate has been thrown down by centrifugation.

It may be concluded that, under the conditions of the experiments:

1. A substance enhancing the proliferative activity of homologous fibroblasts is precipitated from serum by CO_2.

2. The serum from which the precipitate has been removed inhibits the proliferation of homologous fibroblasts more markedly than does the original serum. The solution of the precipitate in the treated serum has about the same effect on fibroblasts as the original serum.

3. The restraining effect of serum on the activity of homologous fibroblasts is due in part to the antagonistic action of growth-activating and inhibiting substances.

We wish to express our thanks to Dr. Albert Fischer for the preparation of the globulins used in a few of the experiments.

SURFACE TENSION OF SERUM.

V. Relation between Time-Drop and Serum Antibodies.

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I.

INTRODUCTION.

As stated in our previous papers,[1,2] the surface tension of serum decreases spontaneously in function of time. When the serum, instead of being pure, is diluted, the phenomenon is more marked and shows a maximum at a certain optimum concentration. This drop of the value of the surface tension, called time-drop for brevity, is due to adsorption in the superficial layer of active substances. The question arises whether certain modifications of the serum, detected thus far only by biological reactions, will not affect these substances and, consequently, the time-drop.

The value of the surface tension expresses the algebraic sum of the elementary interactions of the molecules and micellæ composing the serum, attractions and repulsions. By diluting the serum considerably, it is probable that, at a given concentration, an organized monomolecular, or more likely monomicellar layer is obtained at the surface of the liquid. In this layer, the molecular and micellar interactions will be maximal owing to the fact that, being no longer piled up and irregularly distributed, they are all submitted to symmetric forces in the same plane. In this plane, the summation of these forces can take place unhindered so as to produce an enhanced effect on the time-drop. This hypothesis is confirmed by the existence of the maximum time-drop at a dilution of 1:10,000. It is possible

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707.

[2] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxvi, 115, 547.

that any modification, not only chemical (of the molecules), but also structural (of the micellæ), must manifest itself by a change in the value of the individual field of force of every micella, which, in turn, will make itself perceptible by a change in the integral phenomenon, the surface tension. The study of the action of heat on the surface tension of diluted serum seems to support this assumption, although our ignorance concerning the structure of serum does not always allow an accurate explanation of the observed facts.

The purpose of the present work was to find out whether the properties conferred upon a serum by immunization can be detected by another method than the biological one; namely, by observations of the surface tension of this serum.

The so called miostagmin reaction worked out by Ascoli,[3] and by many others since, deals with a similar problem, but with a fundamental dissimilarity in procedure. Ascoli measured the surface tension of a solution of immune serum, to which an alcoholic solution of its antigen was added, immediately thereafter and at the end of 2 hours of incubation; then he measured the surface tension of a solution of normal serum to which the same alcoholic antigen had been added. The differences observed could, according to the author, be used to determine whether or not a serum contained immune bodies. The reaction has been thoroughly reviewed and investigated recently by Gouwens.[4] It appears from this paper that it cannot be relied upon, at least in the case checked by him, in which *B. paratyphosus* B was employed as antigen.

II.

EXPERIMENTAL.

The technique of the measurement of surface tension has been dealt with extensively in preceding papers.[1,2] We will therefore confine ourselves to the technique used in the preparation of animals and solutions.

Thirty-one rabbits, four dogs, and eighteen chickens were used. From 15 to 20 cc. of blood were taken before the injection of antigen, and the same amount after 15 days, when immunity had developed. For certain series of experiments, the animals were bled practically every day in order to follow the course of the reaction. Control

[3] Ascoli, *Münch. med. Woch.*, 1910, lvii, 62.

[4] Gouwens, W. E., *J. Infect. Dis.*, 1922, xxxi, 237.

animals were bled at the same time. The antigens used were sheep cells, dog serum, and egg white. A few rabbits were injected with turpentine and rabbit cells in order to make sure that the phenomenon was really referable to the immunization process, and not merely to the fact that the animal had been injected with foreign material. In general, four injections of antigen of 4, 6, 8, and 10 cc. respectively were made at 3 day intervals.

Preparation of Antigen.—When sheep or rabbit cells were used as antigen, the blood was received in a sodium citrate solution and centrifuged. The cells were then washed three times in isotonic salt solution, centrifuged again, and the normal volume of blood made up with saline.

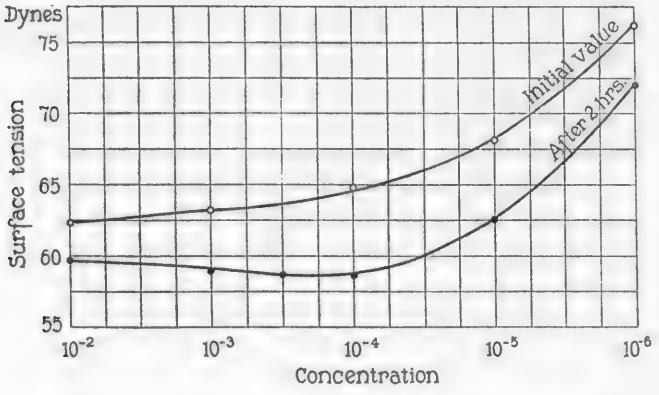
Preparation of Serum Solutions.—All the vessels used in the experiments were carefully cleaned according to the technique previously described.[1] The serum was diluted with a saline solution the surface tension of which at 23°C. was 76.2 dynes ± 0.2. Solutions were made for the first experiments at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Later on, only 10^{-4} solutions were used, for a reason which will become obvious to the reader.

Measurements of Surface Tension.—Measurements were made with the du Noüy tensiometer, according to the technique described in former papers,[1] and the time-drop in 2 hours was observed. All measurements were made at 23°C. ± 0.5.

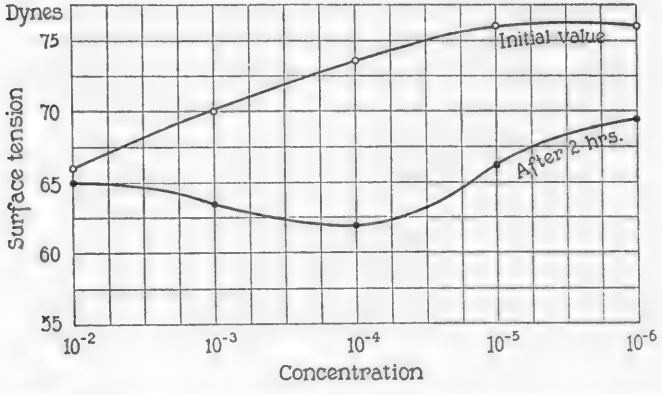
Control.—The state of immunization of the animals was ascertained whenever the time-drop was measured by tests of the sera for the expected hemolysin and precipitin, which always ran parallel.

The first series of experiments was carried out with solutions of different concentrations, in order to check up the assumption made in the first part of this paper, namely that, should a modification be detected after immunization, it must be maximum at a dilution of 10^{-4}. The results of one experiment are shown in Text-figs. 1 to 3. For all sera studied, two curves were drawn, but the results are clearer in Text-fig. 3, in which only the differences between the initial value of the surface tension and the value after 2 hours have been plotted. For convenience, these differences have been given the sign +. In other words, Text-fig. 3 expresses the values of time-drop, $d = \gamma_o - \gamma$, in function of the concentration, γ_o being the surface

tension at the time o, and γ that of the same sample of liquid 2 hours later.



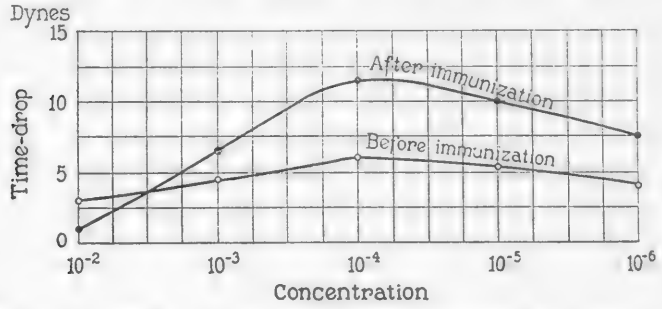
TEXT-FIG. 1. Values of the surface tension of serum solutions before immunization. Serum 121 (rabbit).



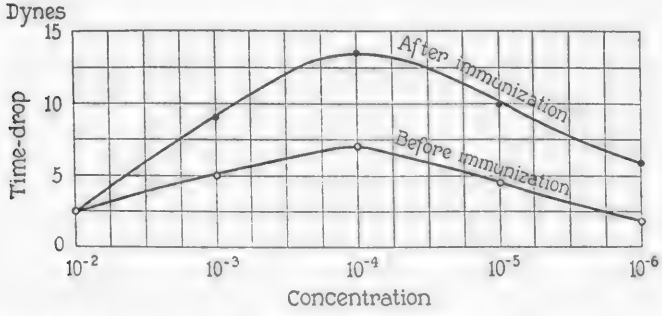
TEXT-FIG. 2. Values of the surface tension of serum solutions after immunization (15th day). Serum 121 (rabbit).

It is plain that, in this case, a marked increase of the time-drop was observed, the difference being almost 100 per cent. The results of another set of experiments on seven rabbits are gathered in Text-fig. 4, in which the curves express the mean values.

With chickens, the phenomenon was less striking, the time-drop being smaller. Nos. 1 to 6 showed a mean increase of 4.1 dynes at a dilution of 10^{-4}, instead of the 6.5 dynes shown by the rabbits. The six controls not only manifested no increase, but even a small decrease, (mean value -2.2 dynes). A mere glance at the curves representing



TEXT-FIG. 3. Time-drop curves; *i.e.*, differences between the initial surface tension and the surface tension after 2 hours. Normal and immune serum (No. 121; rabbit). The time-drop of the immune serum is nearly double that of the normal serum.



TEXT-FIG. 4. Time-drop before and after immunization. Mean curves expressing the result of experiments on seven rabbits (Nos. 1 to 7).

the above experiments makes it plain that, as was foreseen, the maximum drop occurs at the concentration of 1:10,000. Consequently, it was thought unnecessary to resort any longer to the tedious and time-consuming series of measurements at all concentrations and in the later work only one solution was prepared, that of 10^{-4}. Fur-

thermore, instead of taking only two sets of measurements, one before immunization and the second after an empirically chosen period of time, the progress of the process of immunization was studied with blood specimens taken at frequent intervals. The results are shown in Table I. Text-fig. 5 represents the mean values of the time-drop in the course of the observations, from the 1st to the 18th day. The normal curve (non-immunized) represents the mean values of three controls, instead of the two given in the table (Rabbits 8 to 10). The curves show plainly the progressive increase in the time-drop after

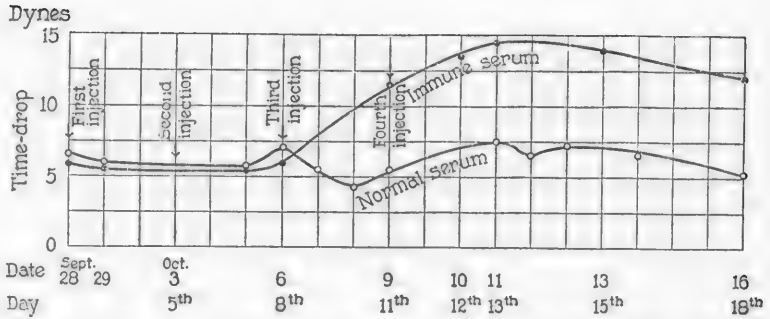
TABLE I.

Value of the Time-Drop of the Serum of Six Rabbits during the Process of Immunization and of Two Controls.

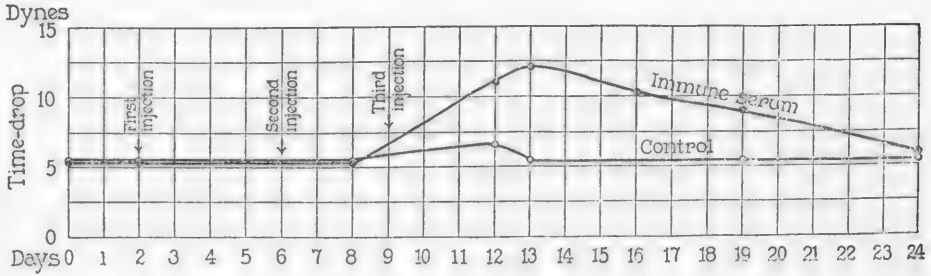
Date.	Controls.		Rabbits injected with sheep cells.			Rabbits injected with egg white.			Remarks.
	No. 9.	No. 10.	No. 11.	No. 12.	No. 13.	No. 14.	No. 15.	No. 16.	
1922	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	
Sept. 28	4.5	6.0	8.0	7.7	6.2	4.2	5.2	4.5	First injection.
" 29	3.0	5.5	6.0	7.0	10.0	8.0	10.0	12.5	
Oct. 2	4.5	2.0	9.0	7.5	10.5	9.5	9.0	6.0	
" 3	4.5	5.0	5.2	7.5	6.5	4.5	7.0	5.0	Second injection.
" 4	2.5	2.0	4.5	4.5	4.0	3.5	4.5	7.5	
" 5	1.5	3.0	9.0	10.0		8.0	4.0	12.0	
" 6	1.8	6.2	9.0	12.5	11.0	12.7	13.0	10.8	Third injection.
" 9	5.0	11.0	10.5	13.0	7.5	5.5	6.5	10.5	Fourth "
" 10	5.5	7.0	11.0	12.0	17.5	11.5	14.0	15.0	
" 11	8.0	7.0	14.5	13.0	11.0	15.5	17.3	17.5	
" 13	5.5	7.0		14.8	13.5	13.5	14.0	14.0	
" 16	3.0	5.5	Died.	11.0	13.5	13.5	12.5	10.5	

the 8th day, and a maximum on the 13th day, 2 days after the last injection. The subsequent decrease of the immunity was also studied. Text-fig. 6 shows the evolution of the phenomenon until the 24th day. This curve is exactly similar to those obtained by immunity reactions in the test-tube.[5] Another experiment was made with eight normal rabbits and carried on for over 3 weeks in order to obtain an absolute control. The results, in the form of a mean curve, are likewise given in Text-fig. 6.

[5] Jörgensen, A., and Madsen, T., Festskrift ved Indvielsen af Statens Serum-Institut, Copenhagen, 1902, Paper 6, p. 12. Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 535.



TEXT-FIG. 5. Curve representing the evolution of the process of immunization. Mean values of six experiments on rabbits and three controls (Table I). Only the time-drop of serum diluted to 1:10,000 was studied.



TEXT-FIG. 6. Curve representing, as does Text-fig. 5, the evolution of the process of immunization in a rabbit (No. 17) up to the 24th day. The control curve represents the mean values of the time-drop of eight sera.

The question arose as to whether the increase in time-drop was really due to actual immunity or simply to the introduction of a foreign substance in the circulation. In other words, was it a consequence of the reaction following the introduction of foreign substances, irrespective of their nature, or was it referable to specific antibodies developed in the animal as a result of the injection of antigen? In order to answer this question, two rabbits were injected with rabbit cells, and two with turpentine. The time-drops are given in Table II. Text-fig. 7 expresses the results of the two groups of animals respectively, as compared with the control. Text-fig. 8

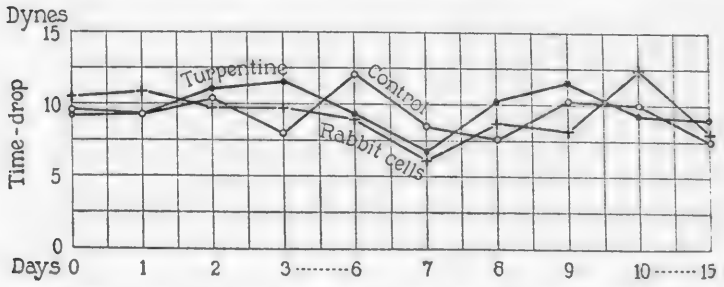
TABLE II.

Value of the Time-Drop of the Serum of Four Rabbits Injected with Homologous Cells and Turpentine and of One Control.

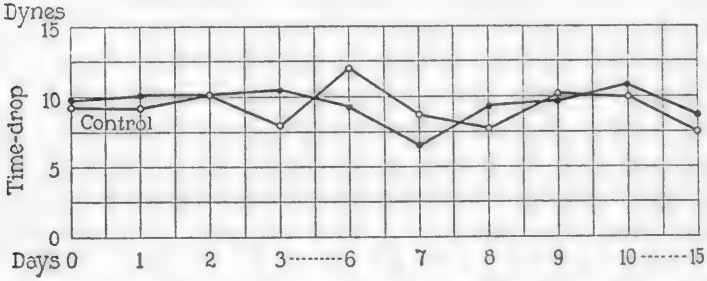
Date.	Control.	Rabbits injected with rabbit cells.		Rabbits injected with turpentine.	
	No. 8.	No. 18.	No. 19.	No. 20.	No. 21.
1922	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*
June 20	9.5	10.5	10.5	10.5	8.0
" 21	9.3	11.8	10.0	11.8	7.0
" 22	10.3	9.5	9.3	12.0	10.1
" 23	8.0	8.2	11.4	12.7	10.0
" 26	12.0	10.0	7.7	11.7	7.7
" 27	8.5	6.8	5.3	5.2	9.0
" 28	7.7	8.3	9.0	10.0	10.8
" 29	10.3	7.0	9.5	12.3	11.0
" 30	10.0	11.5	13.8	10.0	8.8
July 5	7.5	8.5	7.5	4.8	13.2

gives the mean curve representing the four sets of measurements. It is manifest that a greater number of experiments would only tend to flatten the curve, which runs parallel to the axis of time. There is no tendency to a change of level or a maximum as in the experiments reported in Table I and Text-fig. 5, and the controls of the latter, although of higher absolute value, are equally parallel to the axis. These results were checked with four dogs injected with turpentine; two gave a slight increase of the time-drop (Nos. 1 and 2), and two a decrease (Nos. 3 and 4). In this case, likewise, there was no sign of constant increase. Consequently, it is probable that the increase

in the time-drop observed in all animals injected with heterogeneous cells and sera, or foreign proteins (egg white), is due to the formation of new substances (antibodies) or to a relatively permanent modification of some of the elements of the serum. An attempt was made to



TEXT-FIG. 7. Curve representing the results of an experiment similar to those expressed by Text-figs. 5 and 6, but in which four rabbits were injected with turpentine and homologous (rabbit) cells (Table II). There is no indication of a constant increase in the time-drop, as no antibodies were formed.



TEXT-FIG. 8. Curves representing the same results as Text-fig. 7, except that the curve here represents the mean value of all four experiments. The tendency toward flattening is obvious.

determine whether the modifications would affect the refractive index. It seemed not unlikely that a change in the serum which acted on the surface tension so importantly, that is to say, the stray fields of the group molecules, would also affect the optical properties. However, such was not the case, as Table III shows.

TABLE III.

Refractive Indices of Immune and Normal Sera the Time-Drop of Which Is Reportea in Table I.

Date.	Controls.		Rabbits injected with sheep cells.			Rabbits injected with egg white.		
	No. 9.	No. 10.	No. 11.	No. 12.	No. 13.	No. 14.	No. 15.	No. 16.
1922								
Sept. 28	1.3438	1.3429	1.3442	1.3422	1.3440	1.3433	1.3438	1.3429
" 29	1.3398	1.3392	1.3399	1.3385	1.3402	1.3390	1.3407	1.3325
Oct. 2	1.3398	1.3386	1.3409	1.3390	1.3408	1.3397	1.3410	1.3381
" 3	1.3440	1.3387	1.3404	1.3398	1.3405	1.3405	1.3420	1.3390
" 4	1.3388	1.3384	1.3395	1.3388	1.3400	1.3398	1.3399	1.3392
" 5	1.3398	1.3393	1.3408	1.3350		1.3395	1.3401	1.3385
" 6	1.3422	1.3415	1.3415	1.3410	1.3420	1.3440	1.3415	1.3420
" 9	1.3415	1.3413	1.3401	1.3420	1.3402	1.3405	1.3411	1.3405
" 10	1.3400	1.3388	1.3391	1.3402	1.3403	1.3403	1.3398	1.3391
" 11	1.3391	1.3385	1.3391	1.3383	1.3396	1.3396	1.3390	1.3386
" 13	1.3399	1.3385	Died.	1.3385	1.3383	1.3390	1.3391	1.3391
" 16	1.3389	1.3385		1.3370	1.3390	1.3384	1.3392	1.3390
Mean average....	1.3400		1.3400					

III.

DISCUSSION.

Our knowledge of the relation between the chemical structure and properties of organic compounds, and the forces existing between their molecules or group molecules, when in solution, is too scant to permit an interpretation of the meaning of the value and changes of the surface tension of a liquid. Furthermore, our knowledge of the chemistry and physicochemistry of antibodies is practically nil. Therefore, the meaning of the experimental observations gathered in this paper remains to be discovered. But one thing may safely be said: in certain immunity states, a decrease in the surface energy of the substances normally adsorbed in function of time in the surface layer is observed.

It may be pointed out that this is probably the first direct measurement by physical means of a change in the serum consecutive to immunization. It is not improbable that the method will yield more accurate data than those furnished by biological tests in following, for example, the progress of the process of immunization.

IV.

CONCLUSIONS.

An attempt was made to apply to the study of immune sera the fact, pointed out in our previous papers, that at a given dilution the serum shows a maximum drop of surface tension in function of time (time-drop). The results of the experiments here reported would seem to indicate that:

1. The time-drop of a serum solution is greater after the animal has been immunized than before immunization (from 50 to 100 per cent).

2. The time-drop is maximum at a dilution of 1:10,000, as had been predicted from the data previously acquired.

3. The size of the molecules or micellæ is probably unchanged after immunization, since the maximum drop is not shifted then.

4. Measurements made during the process of immunization showed that the time-drop increases after the 8th day and second injection, and reaches a maximum toward the 13th day, while the controls undergo no increase.

5. The injection of substances non-productive of antibodies, such as homologous cells or turpentine, does not result in the production of a permanent maximum. This shows that the maximum observed when heterologous cells are used is due to the presence of antibodies, and not to the mere injection of foreign material.

6. The measurement of the refractive index of the sera failed to demonstrate any differences between the sera of the controls and those of the experimental animals.

THE ABSORPTION OF SPECIFIC AGGLUTININS IN HOMOLOGOUS SERUM FED TO CALVES DURING THE EARLY HOURS OF LIFE.

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In a former communication[1] it was shown that calves fed with cow serum in place of colostrum soon after birth and receiving at the same time the serum into a vein and the subcutis survived and developed normally. Calves which received the serum in the milk only did not fare so well.

To supplement these data the work to be reported was undertaken.

Four calves were fed with serum, the first, small quantities in the milk, as were those of the former experiment, the others one large dose without milk. At the same time the serum of each calf was tested to trace the absorption of agglutinins through the digestive tract as was done when a high titer colostrum was fed.[2] The experiment on the absorption of agglutinins was extended to include two new-born calves fed on a high titer milk in place of colostrum and two other calves fed on a high titer serum when they were more than 3 days old. The cow serum used was drawn from a cow (No. 600) treated with living cultures of *Bacillus abortus* at intervals to bring about a relatively high agglutinin titer. The serum fed to the two first calves (Nos. 976 and 980) had a limiting titer of 1:2,560. The clumping was complete and the suspending fluid cleared at 1:160. The serum fed Nos. 988 and 993 had a limiting titer of 1:5,120. The fluid was cleared at 1:640. The milk from Cow 938 fed to calves after the experimental feeding of serum or of a special milk of high titer failed to show any clumping at 1:20. In all cases the agglutinin

[1] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 453.

[2] Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.

titers refer to *Bacillus abortus*. The figures given refer to the limiting titer; *i.e.*, the dilution in which some clumping is still recognizable. In the tables, C. stands for complete clumping and clearing of the fluid. The tests were carried out well beyond the limit of the titer but they are omitted from the tables. The temperature of the calves was taken three times each day. Only significant figures are quoted.

EXPERIMENTAL WORK.

No. 976.—Third calf of a Holstein cow. Male, born Sept. 22, 4.45 p.m. A sample of 30 cc. of blood drawn at 5.20 p.m. Calf dried by gently rubbing with towels. At 5.30 p.m., fed about 70 cc. of serum from Cow 600, to which milk from Cow 938 was added to a total of about 375 cc. Fed from bottle. At 11.30 p.m., a second sample of 30 cc. of blood was taken. Calf passing dry, firm pellets of rectal meconium. Fed serum and milk as before. Sept. 23, 8 a.m. About 70 cc. of blood drawn and calf fed same mixture of serum and milk. At 1 p.m., 25 cc. of blood drawn. Calf takes 3 pounds of milk. Meconium voided. Fed 3 pounds of milk at 5 p.m. Sept. 24. Fed 3 pounds of milk three times today. 25 cc. of blood drawn at noon. Sept. 25. Fed in all 11 pounds of milk and bled 65 cc. of blood. Sept. 26. Fed 11 pounds of milk. Sept. 27. Fed 11 pounds of milk. From now on to Oct. 10, when the animal was killed, no abnormal symptoms were noticed. The autopsy was negative throughout.

TABLE I.

No. 976, Fed Cow Serum of High Titer.

Age of calf.	Fed serum.	Serum dilutions.						Control.
		1:5	1:10	1:20	1:40	1:80	1:160	
	cc.							
45 min.	70	−	−	−	−	−	−	−
6 hrs., 45 "	70	+++	++	+	+	−	−	−
16 " 15 "	70	+++	++	+	+	±	−	−
21 "		C.	+++	++	++	+	−	−
42 " 50 min.		+++	++	+	+	+	−	−
2 days, 16 "		+++	+	+	+	−	−	−
18 "		C.	++	+	±	±	−	−

Table I shows the absence of agglutinins in the blood before any food had been taken. 6 hours after 70 cc. of serum had been fed there was a distinct indication of clumping at 1:40. After two additional doses, the last 16 hours after birth, there was an increase to 1:80. When the calf was 18 days old, the agglutinins were still present but diminished in amount.

No. 980.—Bull calf of a Holstein cow (first since purchase), born Sept. 27, 11.30 a.m. Weight 80 pounds. Calf born into manure drop while cow was in stanchion. Some mucus in upper air passage of calf when found. At 12 noon, after the withdrawal of 25 cc. of blood, the calf was fed from a bottle about 500 cc. of warmed serum of Cow 600, then dried by gentle friction with towels. At 6.30 p.m., after 25 cc. of blood had been taken, the calf received its first meal of 2 pounds of milk (Cow 938). Sept. 28. Fed three times with 9 pounds of milk in all. 25 cc. of blood withdrawn at 9.15 a.m. Sept. 29. Fed same as yesterday. Sept. 30. Slight elevation of temperature and softening of feces towards evening. Oct. 1. Feces quite soft. Temperature 39.6°C. in evening. Oct. 2. Feces now liquid. Temperature 38.8°C. in evening. Takes food normally. Oct. 3. Takes 11 pounds of milk. Feces less fluid. Oct. 4. Calf improving generally. Feces more consistent. Oct. 5. Calf now quite active. Killed. Autopsy negative.

TABLE II.

No. 980, Fed One Dose of 500 Cc. of Serum of Cow 600 Soon after Birth.

Age of calf.	Serum dilutions.								
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
40 min.	−	−	−	−	−	−	−	−	−
6 hrs., 50 "	C.	C.	C.	++++	+++	++	+	+	−
21 " 45 "	+++	+++	+++	++	++	++	+	−	−
8 days.	+++	+++	++	++	++	++	+	−	−

Table II shows that the blood serum of the calf at birth did not agglutinate *Bacillus abortus* in a dilution of 1:5. 6 hours after feeding serum, the titer was 1:640. The titer maintained itself at a slightly lower level until the calf was killed.

No. 988.—Holstein bull calf, born Oct. 20, 3.30 p.m. Weight 77 pounds. Slight traction applied during delivery. Sample of 25 cc. of blood withdrawn and calf fed at 4.12 p.m. with serum of Cow 600 from a bottle. About 650 cc. taken. At 10.15 p.m. another sample of about 40 cc. of blood withdrawn and calf fed from a bottle about 2 pounds of milk (Cow 938). Oct. 21. Calf takes about 9 pounds of milk in three doses. About 25 cc. of blood taken. Calf at end of day is normal and free from diarrhea. Oct. 22. Milk taken as before. Feces semifluid at noon. Oct. 23. Slight elevation of temperature. Calf takes food as usual. Oct. 28. Calf has been normal to date. A sample of 30 cc. of blood taken. Nov. 1. Weight 82 pounds. Killed by stunning and bleeding. Autopsy entirely negative.

TABLE III.

No. 988, Fed One Dose of 650 Cc. of Cow Serum within 1 Hour after Birth.

Age of calf.	Serum dilutions.									Control.
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
40 min.	−	−	−	−	−	−	−	−	−	−
6 hrs., 45 "	C.	C.	C.	C.	++++	+++	++	+	−	−
17 " 5 "	"	"	"	"	+++	++	++	+	−	−
8 days.	+++	++	++	++	+	+	+	+	−	−
12 "	++++	++++	+++	++	+	+	−	−	−	−

Table III shows that the serum of the calf was negative at 1:5. 6 hours after the cow serum had been fed, the blood titer had risen to 1:640.

No. 993.—Holstein bull calf, born Oct. 26, 1.20 p.m. Weight 105 pounds. Calf dropped into manure drop. Chilled by exposure. A sample of blood (30 cc.) withdrawn and calf fed at 3 p.m. from a bottle 500 cc. of serum of Cow 600. At 9.25 p.m., another sample of 25 cc. of blood taken and calf fed about 2 pounds of milk (Cow 938) from a bottle. Oct. 27. A sample of about 50 cc. of blood taken. Calf begins to scour. Takes about 9 pounds of milk in all. Oct. 28. Calf not scouring early, but later feces again watery. Oct. 29. Takes 9 pounds of milk. Diarrhea continues. Calf ill. Oct. 30. Temperature around 39°C. Diarrhea continues. Takes about 9 pounds of milk. Nov. 1. Feces now more consistent. Temperature 39.7°C. at 5 p.m. Indications of pneumonia. Nov. 2, 5 a.m. Temperature 40°C. Calf weak and stiff in gait. Takes only half its food. Nov. 3. Temperature 40.6°C. in evening. Has taken only about half its allowance of milk. Respirations rapid with increased abdominal movement. Nov. 4. Temperature 40.8°C. at 11 a.m. Calf brighter and takes the usual amount of milk. Is able to stand alone. Nov. 5. Temperature fluctuates around 40°C. Breathing abdominal in type. Calf takes 12 pounds of milk in three meals.

Nov. 21. The history of this calf since the former date is one of gradual improvement. The temperature remained around 40°C. for some days and gradually fell to about 39°C. The respiration remained rapid and the type abdominal. Auscultation indicated pneumonia. The calf continued to take its food regularly and during the past week took 15 pounds of milk daily. Killed today by cutting neck vessels after stunning with a blow.

The autopsy revealed total necrosis of the right ventral lobe of the lung. One-half of the adjacent portion of the large caudal lobe was similarly involved but the

necrotic masses were isolated and easily pulled out of their respective cavities. The whole was walled off by a thick membrane involving the epicardium and the right wall of thorax. The cavity so formed contained 800 cc. of a thick, chocolate-colored liquid. A small necrotic focus was found at the root of the left ventral lobe. The remaining lung tissue was variegated with areas of collapse. The stump of one umbilical artery had on its sheath a tumor-like body 1.5 to 2 cm. in diameter consisting of a thin capsule and cheesy, purulent contents. Owing to the mixture of bacteria present in the necrotic lung tissue, the etiological factor remains unknown. Pneumonia prevailed in the herd at the time.

TABLE IV.

No. 993, Fed One Dose of 500 Cc. of Cow Serum of High Titer about 2 Hours after Birth.

Age of calf.	Serum dilutions.								Control.
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	
1½ hrs.	−	−	−	−	−	−	−	−	−
8 "	C.	C.	++++	+++	++	+	±	−	−
20 "	"	++++	+++	++	+	+	+	−	−
11 days.	++++	++++	+++	+++	++	+	+	−	−
23 "	++++	+++	+++	+++	++	++	+	−	−

The absorption of agglutinins from the blood serum is shown in Table IV. Within 7 hours the titer of the calf's serum rose from less than 1:5 to 1:160 and maintained itself at nearly this level until the calf was killed.

For the purpose of determining whether agglutinins are absorbed from a high titer milk, not colostrum, two calves soon after birth were fed with milk from a cow (No. 799) infected spontaneously with *Bacillus abortus* and delivered of a premature calf about 4 months previous. The agglutination of *Bacillus abortus* by the milk during the period in which it was used on the calves was complete at 1:160 and just recognizable at 1:1,280.

No. 992.—Holstein bull calf, born Oct. 25, 1.15 p.m. Weight 97 pounds. It was immediately removed and dried with towels. Milk of a high titer (Cow 799) was fed at 5 p.m. Before the first meal of 850 cc. of milk, about 50 cc. of blood were withdrawn and about 25 cc. at 11 p.m. At this time the calf was fed 940 cc. of the same milk, refrigerated since the first meal and warmed to 36°C. Oct. 26, 6 a.m. Calf bled (about 50 cc.) and then fed 3 pounds of freshly drawn milk from the same cow. Calf bled again (25 cc.) at 11.20 a.m. and fed 2 pounds of

milk from the same cow. Diarrhea started late in the day. A third meal of about 2 pounds of milk fed in the evening. The calf is distinctly ill at this time. Oct. 27. From now on, the calf is fed milk of low titer. It takes about 9 pounds in all today. Condition improved. Oct. 28. Calf improving. Takes milk as usual.

Nov. 3. Calf has been normal since Oct. 28. Takes about 12 pounds of milk. Nov. 7. Calf has been in normal condition since preceding date. On this day, when 13 days old, it was fed, after withholding the morning meal of milk, with 520 cc. of serum of Cow 600, having a high agglutinating titer towards *B. abortus*. The very slight rise in the blood titer of the calf following this feeding is within the margin of error of the method.

Nov. 10. Calf killed and autopsied. Organs normal.

TABLE V.

No. 992, Fed Milk of High Agglutinin Titer Five Times, First Meal When 4 Hours Old, Last Meal When 34 Hours Old.

Age of calf.	Serum dilutions.						Control.
	1:5	1:10	1:20	1:40	1:80	1:160	
3½ hrs.	−	−	−	−	−	−	−
10 "	++++	++	+	−	−	−	−
17 "	++++	+++	+	±	−	−	−
22 "	++++	+++	+	±	−	−	−
44 "	+++	+	+	−	−	−	−
12 days.	+++	+	±	−	−	−	−
13 "	Fed 520 cc. of serum (Cow 600) with high titer.						
13 " 6¼ hrs.	++++	++	+	−	−	−	−
13 " 23½ "	+++	++	+	±	−	−	−

The results of feeding milk and later serum of a high agglutinin titer to this calf are presented in Table V. It will be noted that 6 hours after the first of the three meals of this milk fed on the 1st day of life there was a distinct rise in the blood titer of the calf from less than 1:5 to 1:20. Two later meals produced only a doubtful rise beyond this. On the 12th day it had fallen to 1:10. It is to be noted that this calf survived without colostrum.

No. 999.—Holstein bull calf, born Nov. 7, 8.40 a.m. Weight 86 pounds. Umbilical cord was torn away at its insertion. Removed to isolation unit immediately, a sample of 50 cc. of blood withdrawn, and the calf dried by gently rubbing with bath towels. Calf barely able to stand.

Milk with high agglutinin titer (Cow 799) was collected at 5.10 p.m., Nov. 6, and refrigerated until next morning. It was then gently warmed to 37°C. and 700 cc. fed to this calf from a bottle at 9.40 a.m. Fed 912 cc. from the same lot of refrigerated milk, warmed as before, at 4 p.m., and again 850 cc. at 10 p.m. During the day a sample of 20 cc. of blood was withdrawn at 3.50 p.m. and another of 15 cc. at 9.50 p.m.

Nov. 8. Fed 3 pounds of milk from Cow 938 (low titer) at 5 a.m., 11 a.m., and 5 p.m. During the morning diarrhea set in and continued, stools becoming watery. Temperature 39.2°C. in the evening.

Nov. 9. Calf now very weak, unable to stand. Diarrhea continues and the temperature rises to 41.1°C. in the evening. Refuses food after the morning meal. Lying down and comatose towards evening. Died about 8 p.m. Body cooled with a stream of water and refrigerated.

Nov. 10. The autopsy showed the following. Umbilical stump absent. Umbilical vein closed and filled with a dark, firm clot. Hemorrhagic infiltration under serosa of stumps of umbilical arteries, extending to cephalic part of bladder. The rumen contains milky fluid and the fourth stomach both fluid and a firm lump of casein about 8 cm. in diameter. The mucosa appears as if covered with a thin, veil-like membrane which comes away in patches and is made up of desquamated epithelium. Mucosa punctuated with faded petechiæ. The mucosa of small and large intestines has deeply congested areas. Trachea cyanotic. Lungs slightly edematous and hypostatic. Epicardial vessels distended and forming hemorrhagic elevations at base of large vessels. All heart cavities filled with soft, dark clots. Blood in general surrounding clots is thick. Kidneys congested throughout. Liver cells contain considerable orange pigment but no fat globules. Films from spleen pulp show a fair number of colon-like bacilli but no other types. Cultures made with heart's blood, spleen, liver, and kidney tissue develop large numbers of colonies of *B. coli communis*. No other forms present.

TABLE VI.

No. 999, Calf Receiving Milk with a High Agglutinin Titer in Place of Colostrum.

Age of calf.	Serum dilutions.					Control.
	1:5	1:10	1:20	1:40	1:80	
1 hr.	−	−	−	−	−	−
6 hrs.	+++	+++	+	−	−	−
12 "	++++	++++	++	+	−	−
25 "	++++	++	+	−	−	−

Table VI shows absence of agglutinins from the blood of the calf at birth at 1:5 and a rise to 1:40 when it was 12 hours old.

One calf was treated as a control by feeding ordinary milk, instead of colostrum or serum.

No. 981.—Bull calf, born of a Holstein cow Oct. 7, 3.15 p.m. Owing to large size of calf, traction was necessary to deliver it. At 4.05 p.m. a sample of blood taken and the calf given its first meal of 1½ pounds of milk (Cow 938). Calf weak and unable to stand. Dried off by gentle friction with towels. Voids large amount of meconium towards midnight. Fed 1½ pounds of milk from a bottle at this hour. Oct. 8. 85 cc. of blood withdrawn at 11.45 a.m. Temperature 39.8°C. towards evening. Calf begins to scour. Oct. 9. Diarrhea continues. Feces watery. Calf weak with unsteady gait. Takes 9 pounds of milk today. Temperature between 39° and 39.5°C. Oct. 10. Feces less watery. Takes in all 9 pounds of milk. Oct. 11. Calf less active. Maximum temperature 40.2°C. Gait stiff. Feces soft. Umbilicus tender and slightly swollen. Takes 9 pounds of milk in all. Towards night stiff in all joints. Scarcely able to stand. Oct. 12. Conditions about as yesterday. Feces soft with fetid odor. Stiffness of gait increased. Oct. 13. Temperature 39.4–40.1°C. Takes milk as usual. Oct. 14. Feces soft, orange-colored. Respiration increased with mucous discharge from nostrils. Umbilicus still moist. Calf has to be helped up to take food. Oct. 15. Died during the night. Body chilled with stream of cold water and refrigerated.

Oct. 16. The following conditions noted at autopsy. Superficial necrosis of skin area covered by umbilical cord and edema of underlying tissues. Umbilical vein closed and filled with a dark red clot. Peritoneal surface at this point covered with a thin fibrinous film. Fourth stomach contains about 500 cc. of clotted milk. Mucosa of a washed dark red color. Patches of congestion in small intestine. Lungs slightly edematous. Foci of collapse and congestion, one to several lobules in extent, scattered through all lobes. Pericardial sac contains some turbid coagulable fluid and flakes of fibrin. Epicardium congested and hemorrhagic.

Various joints (knee, elbow, metacarpal, metatarsal, and atlantoodontoid) contain a turbid, yellowish fluid and elastic, whitish masses of exudate.

Cultures show large numbers of bacilli subsequently identified as *B. coli communis* in spleen, liver, kidneys, umbilical vein, joints, and pericardial cavity.

At this time three calves, which had received colostrum from the mother, were taken to serve as additional controls. In addition, the first, No. 984b, was fed with the high titer serum of Cow 600 when 3¼ days old to determine whether the period for absorption from the digestive tract had passed.

No. 984b.—Black heifer calf, born Oct. 28, between 3 and 4 a.m. Weight 67 pounds. Calf remained with the dam until 10 a.m., taking colostrum during

this time. At 10.20 a.m. about 70 cc. of blood were withdrawn for tests. Fed 3 pounds of milk (Cow 985) in the evening. Oct. 29. Takes about 9 pounds of milk during the day. Some diarrhea. Oct. 30. About 25 cc. of blood withdrawn. Takes food regularly. Feces now normal. Oct. 31. Fed at 9 a.m. 500 cc. of serum from Cow 600. Next feeding at 4 p.m. with ordinary milk. At this time about 45 cc. of blood withdrawn. Nov. 10. Calf has been normal up to the present. Killed. Organs normal.

TABLE VII.

No. 984b, Colostrum-Fed. One Dose of Cow Serum Fed When Calf Was 3¼ Days Old. Colostrum of Dam with Titer of 1:160.

Age of calf.	Serum dilutions.						Control.
	1:5	1:10	1:20	1:40	1:80	1:160	
6–7 hrs.	C.	C.	C.	++	+	−	−
2¼ days.	"	"	++++	+++	+	−	−
3¼ "	Fed 500 cc. of cow serum.						
3½ "	++++	++++	+++	++	−	−	−
8 "	++++	++++	+++	++	+	−	−

The feeding of a high titer serum when the calf was 3¼ days old failed to raise the then existing titer of the serum, as shown in Table VII.

No. 1008.—Holstein male calf, weighing 90 pounds at birth. When ¾ hour old it is held up and allowed to suckle three quarters of the dam's udder for about 10 minutes, then removed and cared for in the usual way, receiving ordinary milk from Cow 938. The umbilical stump was moist and hence washed with boric acid and partly cut away. Diarrhea did not appear in this calf. It was killed when 15 days old and found normal.

No. 1009.—Holstein female calf, weighing 68 pounds at birth, first calf, delivered with some assistance. Calf too weak to stand. After 20 minutes it was held up and allowed to suckle from three quarters of the dam's udder for 15 minutes, then removed and cared for in the usual way. It received ordinary milk (Cow 938). It was stronger next day. On the 2nd day diarrhea set in and the calf took only a portion of its meal. This continued another day. Thereafter the condition of the calf was normal. It was killed when 13 days old and found normal.

DISCUSSION.

The foregoing protocols may be analyzed with reference to (1) the significance of serum and colostrum in maintaining the life of the calf during the earliest days, and (2) the absorption of agglutinins from

the digestive tract as conditioned by the vehicle containing them and the age of the calf.

1. The conclusions reached in former publications[1,3] concerning the importance of colostrum to the new-born calf are confirmed and extended. Of three controls receiving it, all survived in a normal condition. Of three receiving ordinary milk, two died and one remained normal. Of four receiving serum, all survived but one became affected with pneumonia which resulted in extensive necrosis of lung tissue. It was killed during a period of apparent convalescence when 26 days old. The serum in one was fed in three small doses in milk; in three, it was fed in one dose of 500 to 650 cc. and was readily taken by the calves. The early diarrhea was practically absent in three.

TABLE VIII.

Summary of Experiments.

Case No.	Treatment.	Limiting titer of food.	Agglutinin titer of calf's blood after the first meal.
976	First meal of 70 cc. of serum plus milk.	1:2,560	1:40
980	One " " 500 " " "	1:2,560	1:640
988	" " " 650 " " "	1:5,120	1:640
993	" " " 500 " " "	1:5,120	1:320
992	First " " 850 " " milk of high titer.	1:1,280	1:20
999	" " " 700 " " " " " " "	1:1,280	1:20
984b	" " " colostrum.	1:160	1:80

In the two fatal cases, not receiving colostrum, there was the same *Bacillus coli* septicemia already described. One (No. 999) lived 2½ days, the other 8 days. In the latter, the longer duration of the disease gave time for the development of a multiple fibrinous polyarthritis and pericarditis associated with *Bacillus coli*.

That calves may now and then not only survive but remain in fairly normal condition without colostrum is illustrated by the third control (No. 992).

2. The prompt appearance of specific agglutinins after the ingestion of a high titer colostrum was duplicated in the cases fed with cow serum as shown in Table VIII, which summarizes the individual experiments.

[3] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 181.

The absence of agglutinins at birth has been demonstrated in all cases of the experiments, although in two of the dams the titer of the blood serum was 1:640 and 1:2,560 respectively. This confirms previous conclusions[1,3] that the placenta of the cow is impervious to these immune bodies and that the rare cases in which they have been found may rest upon some experimental or observational error.

Rettger, White, and Chapman,[4] who had claimed in 1918 that calves "react at birth in the same manner as the dams," state that their conclusions were drawn "without particular reference to the early ingestion of colostrum." As this early ingestion is the kernel of the whole matter, their former claim falls to the ground. The dams react because they are actively immune; the calves after having taken colostrum are only passively and temporarily so, hence they do not react precisely as their dams even after having ingested colostrum.

Huddleson[5] attacked the problem of the transmission of antibodies *in utero* and from his five cases draws the conclusion that there "is no relation between the antibody index of the blood of new-born calves and their respective dams." He employed both agglutination and complement fixation tests. His results indicate that agglutinins may be present in the dam and not in the unfed calf or the reverse, *i.e.* agglutinins may be absent in the dam but present in the calf. The results obtained in this Department point to the absence of agglutinins in the calf at birth.

Concerning the absorption of antibodies in homologous serum, a number of communications dealing with the absorption of antitoxins from the digestive tract appeared early in the present century. They deal largely with the absorption of diphtheria and tetanus antitoxin in heterologous sera, with various species of experimental animals and with periods of life later than those dealt with in the foregoing pages. The only one bearing directly on our problem is by Römer,[6] who describes an experiment with a foal 21 days old. The foal received tetanus antitoxin which was secreted into the milk by the dam after subcutaneous injection of antitoxic horse serum and the same antitoxic horse serum added directly to the milk. The latter was not absorbed, while the former was. This experiment leaves us in the dark as to what would have happened if the homologous serum had been fed soon after birth. Römer himself acknowledges that age might be a deciding factor.

In the experiments of Little and Orcutt,[2] antibodies were absorbed from ingested colostrum as late as 21 hours after birth by two calves kept alive on ordinary milk. Further more precise experiments were interfered with for the time being

[4] Rettger, L. F., White, G. C., and Chapman, L. M., *Storrs Agric. Exp. Station, Bull. 108*, 1921, 87.

[5] Huddleson, I. F., *Cornell Vet.*, 1917, vii, 284.

[6] Römer, P. H., *Z. Immunitätsforsch., Orig.*, 1908–09, i, 171.

by the difficulty of keeping calves alive on ordinary milk when colostrum was withheld beyond this time.

Huddleson[7] fed milk naturally infected with *B. abortus* and therefore containing agglutinins to calves for a number of weeks. Agglutinins were very rarely demonstrated in their blood. It is not stated just how many hours after birth this feeding was begun and it is not possible to assimilate his results with those here reported.

No effort was made to give the experiments here reported a quantitative character by estimating the agglutinin units fed and absorbed. They are, however, sufficiently clear-cut and detailed to warrant certain inferences. As shown in Table VIII, the percentage absorption of agglutinins from a high titer milk was relatively lower than from homologous serum. The percentage absorption from colostrum in the last case appears higher than from serum, but as it was not known how much colostrum had been ingested, further experiments are called for in which colostrum is fed from a bottle.

As to the limit of the period during which absorption of antibodies goes on, it may be stated that the blood titer was not increased by feedings after the first meal. These results do not harmonize entirely with the earlier tests reported by Little and Orcutt[2] and it remains to determine whether the difference in the nature of the respective vehicles—serum, milk, and colostrum—may not account for the discrepancy.

The feeding of serum to two calves respectively $3\frac{1}{2}$ and 13 days old was without effect on the blood titer.

The facts presented have certain bearings on practise. The newborn calf should receive its protective meal as soon as possible after birth. This may be fed from a nursing bottle if the animal is too weak to stand up and suckle the dam. When the udder of the dam is diseased or suspected of carrying dangerous infectious agents, such as tubercle bacilli, and the calf worth raising, cow serum may be fed in place of the colostrum.

The protective action of serum appears to remain intact for months if it is properly collected and stored. It should not contain antiseptics until the harmlessness of such agents has been proved. One

[7] Huddleson, I. F., *Mich. Agric. Exp. Station, Techn. Bull. 32*, 1916.

meal of 500 to 700 cc. is protective but it should be reenforced whenever possible by subcutaneous or intravenous injection soon after birth if the serum is clear and free from molds.

CONCLUSIONS.

The administration of cow serum by feeding may replace colostrum. The agglutinins towards *Bacillus abortus* in it are absorbed during the first hours of life in relatively large amounts when compared with those naturally appearing in ordinary milk. It seems probable that colostrum is the most efficient transporting agent of the antibodies. Experiments are under way to put these conclusions on a quantitative basis with special reference to the age of the calf during which the absorption of homologous antibodies is still possible.

STUDIES ON THE TOTAL BILE.

III. On the Bile Changes Caused by a Pressure Obstacle to Secretion; and on Hydrohepatosis.

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In previous papers the differing influences upon the bile of the gall bladder and ducts have been described,[1] and the fact has been emphasized that, in the absence of infection, these influences are responsible for the varied character of stasis bile. The present communication has to do with the changes occurring in the bile when obstruction hinders but does not prevent its flow through the ducts. During the course of observations upon the theme we have been impressed with what is more than an analogy, with an enlightening similarity, between the events following biliary and urinary obstruction respectively; and while reporting upon the effects of a pressure obstacle upon bile secretion we shall take occasion to discuss the larger theme.

A first instance of the bile changes came to us during work for another purpose which need not here be entered into. A balloon of thin rubber, joined to the common duct by a rubber tube and glass cannula, had been placed within the peritoneal cavity of a dog, and the laparotomy wound closed. Once in every 24 hours thereafter the bile that had accumulated in the balloon was drawn off through an outlet tube passing through the abdominal wall. The fact was recognized that adhesions, contracting down about the artificial reservoir for the bile, would eventually put an end to its usefulness. But this took place slowly; and during the days when the pressure of the adhesions gradually interfered with the filling of the balloon, gradual

[1] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47, 75.

but marked changes were noted in the character of the biliary secretion. A synopsis of the protocol follows.

Experiment 1. Dog 14.—A female hound of 12¾ kilos was operated upon and the bile-collecting apparatus introduced. Ether was the anesthetic. To rule out gall bladder influences, the organ was tied off at the neck with a series of stout ligatures after it had been emptied by aspiration. The tube from the common duct to the collecting bag,—a toy balloon holding 150 cc. without stretching of the walls,—was about 10 cm. long and led, not to the end of the bag near the liver, but to the further one with which it was connected by a curved glass tube. The object of the arrangement was to lessen the danger that infection traveling along the foreign surface would reach the duct; and none did. From the other end of the bag a short piece of rubber tubing led to the body surface through a small stab wound in the abdominal wall. It was kept closed with a clamp, save when bile was to be drawn off, and its tip was stoppered with cotton soaked in 5 per cent phenol, and further swathed in phenolated gauze. The laparotomy wound was closed in three layers. It healed promptly and perfectly. Throughout the period of the observations the condition of the dog was excellent, and the bile remained sterile.

Once in every 24 hours the outlet tube was opened. The intraabdominal pressure caused the bile to gush forth rapidly, and emptied the thin walled balloon completely, as proven both by the failure to obtain more bile by aspiration, and by washing the balloon out with sterile salt solution. The bilirubin yield was quantitated by Hooper and Whipple's method, as in our previous work.

For 10 days after operation a bile of ordinary character, yellow-brown and somewhat syrupy, came from the balloon, but on the 11th day, while the amount of fluid was not greatly lessened (58.2 cc. as compared with 86 cc. for the preceding 24 hours) it was far lighter, a pale greenish brown. At this time the body weight was 11½ kilos. Next day, with still less secretion, there was also less pigment (Table I). The fluid was now thin and greenish yellow, as was that of the 13th and 14th days. The urine became icteric; and on the 14th day, when the dog was killed, a slight jaundice of the tissues could be perceived. Prior to evacuation of the bag on this day the pressure within it was measured with the aid of a glass tube of narrow bore, filled with a 5 per cent watery solution of phenol and connected with the outlet tube. The pressure equalled 370 mm., or in other words was at the upper limit of that developing in dogs with total biliary obstruction.[2] The bag was now emptied, the outlet closed, the animal fed as usual, and after the lapse of 5½ hours, a second manometer determination was made. The pressure was now 300 mm. The bag was forthwith emptied again, 5 per cent phenol solution was run slowly into it, and the pressure changes were

[2] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517. Mitchell, W. T., and Stifel, R. E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 78.

followed. None of the fluid entered until a pressure of 130 mm. had been reached, and after 14 cc. had slowly passed in, the pressure had risen to 160 mm. It should be mentioned that some slight pressure is normally maintained within the duct system by the sphincter of Oddi,[3] though how much is not exactly known.

The outlet tube was now clamped, and the animal chloroformed and immediately autopsied. Except for the minor alterations incident to a moderate general icterus of brief duration, no pathological changes were evident anywhere save in the biliary tract. The ligated gall bladder contained merely a little bile-stained mucus; and, as the original connection between ducts and intestine had not been reestablished, the only path of escape for the bile had led into the bag. The lymphatics from the liver, coursing beside the ducts, were filled with a bright yellow fluid. All the large bile channels were greatly distended with a watery content. The common duct measured more than 1 cm. across near the cannula. The collecting tube and balloon were found swathed in dense adhesions, and the latter was bound thereby to the parietal peritoneum. When the adhesions were cut into there was a gush of serous fluid which had been pocketed about the bag, and this was disclosed empty. But practically at once the contents of the ducts above passed into it, and they themselves collapsed. The fluid transferred so abruptly proved to be the phenol solution previously introduced, now stained a pale yellow with bilirubin. Between bag and ducts no obstruction existed.

Sections of the liver showed a beginning biliary cirrhosis such as is consequent on obstruction.

Here was an animal that during 9 days elaborated a rather syrupy, dark bile. Then the secretion began to diminish in quantity, and became thin and greatly less pigmented (Table I). Some lessening in the concentration of cholates was indicated by Hay's test carried out in graded dilution. Manometer measurements indicated the presence of a pressure obstacle to secretion, which was least immediately after the emptying of the bag, but increased to the degree associated with total obstruction as bile accumulated in the apparatus. The large amount of the secretion obtained from the balloon $5\frac{1}{2}$ hours after the first emptying on the 14th day is not without significance in connection with the pressure variations. More than 31 cc. had collected within this short time, as compared with 38.5, 58.6, and 47.7 cc. for each of the three preceding periods of 24 hours; but already, at the end of the $5\frac{1}{2}$ hours, the pressure within the collecting system approached that of total obstruction. One is justified in the belief that there would have been little further

[3] Mann, F. C., *J. Lab. and Clin. Med.*, 1919–20, v, 107.

TABLE I.

Experiment 1. Dog 14. Weight 11½ Kilos.

24 hr. period No.	Bile amount		Bile character.	Bilirubin.		Bile salts. Hay's test positive in a dilution of.	Bilirubin in urine in 24 hrs.	Remarks.
	Total.	Per hr.		Per cc.	Per hr.			
	cc.	*cc.*		*mg.*	*mg.*		*mg.*	
9	79.4	3.3	Light brown; syrupy.	0.49	1.62	1 in 20	None.	97 per cent hemoglobin in blood.
10	86.0	3.6	" " "	0.46	1.64	1 " 15	1.5	
11	58.2	2.4	Pale greenish brown; very syrupy.	0.33	0.79	1 " 15	4.5	104 per cent hemoglobin.
12	38.5	1.7	Greenish yellow; syrupy.	0.2	0.34	1 " 10	5.0	
13	58.6	2.4	" " slightly syrupy.	0.16	0.38	1 " 10	6.1	106 per cent hemoglobin.
14	47.7	1.9	" " " "	0.22	0.42	1 " 10	8.5	Tissue icterus present.
5½ hrs.	31.2	5.7	Pale yellow; thin.	0.13	0.74	1 " 10		

secretion into the bag had emptying of it been deferred until the next day. At autopsy the distention of the ducts was found to be as great as after total obstruction of some days duration, while the presence of tissue icterus attested to a prolonged retention of bile constituents. For some time, probably, bile secretion into the ducts had taken place only during the first part of each 24 hour period.

Another similar instance was observed in which adhesions progressively limited the expansion of an intraperitoneal collecting balloon. Bile changes occurred that were identical with those just described. Experiments of the sort were then discontinued. But in the course of collections over long periods of time into a balloon outside of the body[4] we have repeatedly had occasion to note biliary changes, whenever a collecting cannula was gradually obstructed by a calculus, or,—following escape of the cannula from the duct,—when a fistulous channel was obliterated by scar tissue.

Methods.

The bile specimens from all our animals were stored in the cold, where they remained without much change for many days, as is usual if the secretion contains little mucus. Whenever alterations in the 24 hour yield pointed to a developing obstruction, all the specimens for some time previous were subjected to quantitative cholesterol determinations by the Autenrieth-Funk method, as were those obtained before total obstruction supervened. Similar determinations were also carried out on successive specimens from animals without any obstruction; but beyond serving as controls these yielded no data of importance in the present connection. The bilirubin output was determined each day by Hooper and Whipple's method, according to a technique already described.[5] No exact quantitation of the bile salts was attempted, but Hay's test was employed in graded dilution to learn of their presence and something of their concentration. It was recognized that the amount, of cholates certainly, and perhaps of cholesterol,[6] can be greatly modified by diet changes. Nevertheless, cholesterol determinations seemed warranted in view of the relation of the substance to human gall stones, and the bearing of partial biliary obstruction on the genesis and development of these latter. The results obtained were clear-cut, and can only be attributed to the progressive obstruction.

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[4] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

[5] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

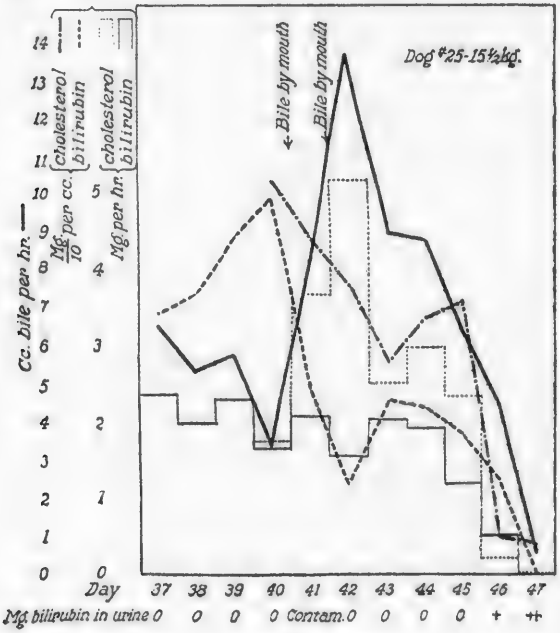
[6] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

It will be recalled that dog bile is poor in cholesterol.[7] The stones which were a frequent cause for obstruction in our animals[4] consisted for the most part of carbonates, with never a trace of the substance.

Typical Instances of Progressive Obstruction.

Some specimen protocols will be given.

Experiment 2. Dog 25. Obstruction by Scar Tissue (Text-Fig. 1).—After 37 days of bile collection from a male terrier of 15½ kilos, the output of the secre-


Dog #25-15½ kg.
← Bile by mouth
Bile by mouth
Cc. bile per hr.
Mg/10 per cc. cholesterol
bilirubin
Mg per hr. cholesterol
bilirubin
Day
37 38 39 40 41 42 43 44 45 46 47
Mg bilirubin in urine 0 0 0 0 Contam. 0 0 0 0 + ++

TEXT-FIG. 1. Changes in the bile during progressive obstruction by scar tissue.

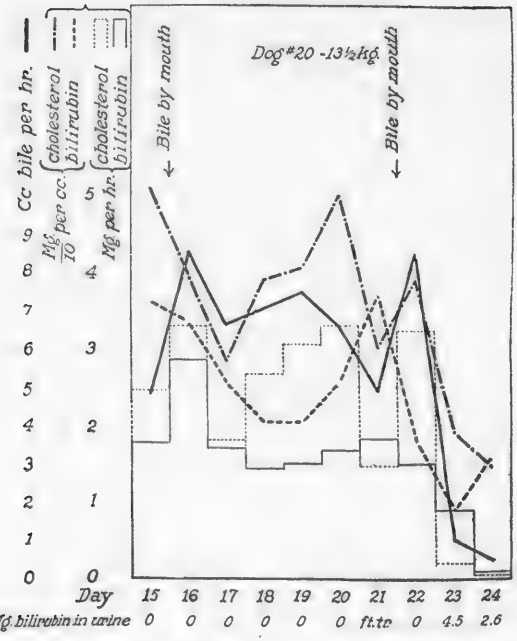
tion, and its pigment content as well, were noted to be growing less, although the animal appeared in good health and spirits. The lessening in pigment continued, despite an intercurrent cholorrhea consequent on feeding sheep bile, and by the 45th day the bilirubin yield had fallen to one-half of the previous "normal,"

[7] Kusumoto, C., *Biochem. Z.*, 1908, xiii, 354; xiv, 416. Fasiani, G. M., *Arch. ital. biol.*, 1915, lxiii, 136.

and on the day following to one-quarter the amount. The fluid was now light green. On the 47th day only a few cubic centimeters of secretion, watery and pale green, was obtained in 25 hours of collection. It held mere traces of pigment and cholesterol (Liebermann-Burchard test), and gave a negative response to Hay's test for cholates. 3 hours later the animal was killed. No further secretion into the collecting bag had taken place. There had been bilirubinuria for 2 days and on the final one a well marked bilirubinemia and a dubious tissue icterus.

Autopsy disclosed the fact that cannula and duct had parted company some time previously. It was recalled in this connection that on the 21st day the dog had pulled upon and opened the outlet tube, thus infecting the bile, though fortunately with a saprophyte harmless to the bile pigment, as incubation tests showed. Probably the cannula was pulled out of the duct at this time. Between the ends of the two there was now a small, gristly lump of scar tissue containing a completely occluded fistulous channel; and the duct system above was distended to the extent usual when obstruction is total. No connection was found with gall bladder or intestine. The distended ducts held a colorless, watery fluid, containing a few glairy, discrete strands, like white of egg. The fluid failed to give Hay's reaction and contained only the faintest trace of cholesterol. The liver lymph was bright yellow with bilirubin. Although infection of the collecting bag had lasted for more than 3 weeks, the hepatic tissue and the fluid in the ducts proved sterile on culture. The organs were normal, except for the changes incident to icterus, and for a slight cirrhosis of the liver of the sort that follows biliary obstruction.

Experiment 3. Dog 20. Obstruction by a Stone (Text-Fig. 2).—On the 18th day of bile collection from a male hound of 13½ kilos the pigment yield was noted to be rather low despite a large fluid output (175 cc.). It underwent a temporary increase to the ordinary amount on 1 of the next 3 days, but thereafter, despite a cholorrhea (to 208 cc.) after bile feeding, it fell off rapidly and so too soon did the fluid yield. On the 23rd day of collection, or the 5th from the time when the changes were first noted, only 23.3 cc. of bile was procured, and this appeared pale green, not yellow-brown as before. Marked bilirubinuria had developed. The 11 cc. of the next 24 hours was somewhat more pigmented. The animal was now sacrificed because its behavior suggested that a bile peritonitis was developing. At autopsy the peritoneal cavity was filled with deeply bile-stained fluid; and a rent in the greatly dilated common duct above the cannula disclosed where the bile had escaped. The cannula was still firmly fixed in the duct, but its lumen had been completely occluded with a calculus. The fluid present high up in the ducts was as brown as normal bile, showing that secretion had promptly reasserted itself after rupture of the duct wall. Probably a little of the new, well pigmented output passed into the bag, for the final fluid therefrom contained, as has just been stated, more pigment than that of the preceding day. All the specimens proved sterile on culture.

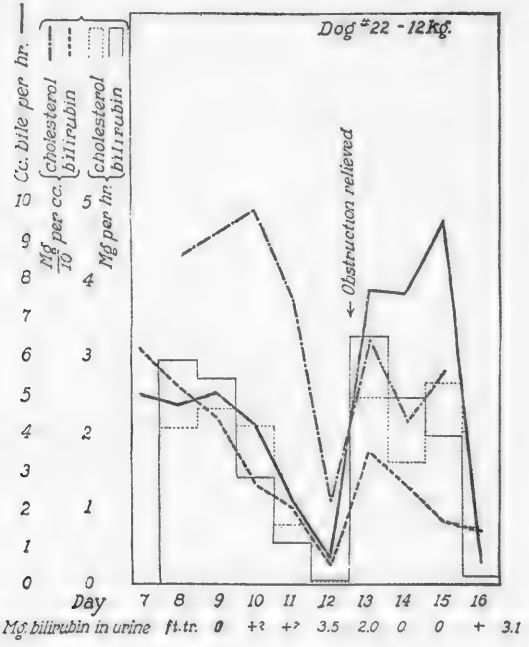

Cc bile per hr.
Mg per cc. cholesterol
bilirubin
Mg per hr. cholesterol
bilirubin
Dog #20 -13½ kg.
← Bile by mouth
← Bile by mouth
Day
15 16 17 18 19 20 21 22 23 24
Mg. bilirubin in urine
0 0 0 0 0 0 ft.to 0 4.5 2.6

TEXT-FIG. 2. Changes in the bile during progressive obstruction by a stone.

Experiment 4. Dog 22. Obstruction by Scar Tissue, Relieved and Recurring (*Text-Fig. 3*).—On the 10th day of bile collection a male bull terrier of 12 kilos yielded somewhat less secretion than usual, with only about half the expected 24 hour yield of bilirubin. On the following day the changes were more outspoken; and on the next, or 12th, a few cubic centimeters only, of very light green, thin fluid, was procured. A marked bilirubinuria was now present. It seemed possible that the trouble might be caused by an abrupt elbow, or kink, in the collecting tube, and with the aim of relieving it, several centimeters of the tube were gently withdrawn from the abdomen. This accomplished the desired effect. During the next few days a dark bile was secreted in relatively enormous amount, and the bilirubinuria disappeared. But soon obstruction developed again, as indicated by the small yield of thin, light-tinted fluid, and it rapidly became total, with result in the development of a marked general icterus during the 3 further days that the animal was preserved, and no bile obtained.

Autopsy disclosed a condition of affairs identical with that of Experiment 2. From first to last the bile was sterile.

Biliary changes like those of the three instances here described (Text-figs. 1 to 3) occurred in all the animals with a progressively increasing obstruction, whatever the cause. The fluid output lessened, and so even more markedly did that of cholesterol and of bilirubin. For not only was the total yield of these substances reduced with the reduction of the fluid output, but the concentration per cubic centimeter as well. The final content of the stopped ducts was a colorless,


Dog #22 - 12 Kg.
Cc. bile per hr.
Mg per cc. cholesterol bilirubin
Mg per hr. cholesterol bilirubin
Obstruction relieved
Day
7 8 9 10 11 12 13 14 15 16
Mg. bilirubin in urine ft.tr. 0 +? +? 3.5 2.0 0 0 + 3.1

TEXT-FIG. 3. Changes in the bile during progressive obstruction by scar tissue.

limpid, "white bile," practically devoid of cholates and cholesterol. At no time as the obstruction developed was there evidence that any of the substances mentioned was secreted in abnormally high concentration; nor was there a thickening of the fluid but always a thinning.

We had planned detailed observations on the character of the bile elaborated against various pressures but were prevented from undertaking them by the difficulty of devising a valve that would precisely

regulate the slight pressure increases compatible with secretion and at the same time control a fluid so variable in consistency as is the bile. Fortunately the progressive obstruction developing in our animals as the result of stones or scarring nearly simulates the clinical occurrence in man.[8]

DISCUSSION.

The bile changes consequent on a pressure obstacle to secretion are the worst possible ones for the body as so much tissue, since they further a retention of biliary constituents instead of aiding elimination in the face of the difficult conditions. But they could not be better suited to meet the grave problem raised by partial obstruction if they had been planned for the purpose. The fluid elaborated by the liver is of the precise sort required in order to avoid the crisis of total obstruction. In previous papers[1] we have emphasized the activity of the gall bladder to make matters worse during partial obstruction of the common duct by so thickening and concentrating the bile above the obstacle that its escape is hindered and calculus formation promoted. The conditions obtaining in the animals of the present work may be likened with reason to those when there is obstruction of the hepatic duct in human beings, or of the common duct when the activities normal to the gall bladder have been abolished by pathological changes. The fact that gall stones situate in the hepatic duct of man cause relatively little trouble has been the subject of frequent comment in the past. One reason is to be found in the alterations that take place in the bile when obstruction impends. In proportion as the pressure obstacle to secretion develops, the bile becomes poorer in the substances that render it viscous or out of which stones form and enlarge. Furthermore, during the intervals when the bile can escape with ease, the accumulation of its constituents within the body is voided, not in a fluid heavily charged with them, but in one so copious that their concentration is actually less than the normal.[5]

It may be objected that the gall stones which were the agent of obstruction in some of our animals enlarged despite the circumstance that the character of the biliary fluid seemed unfavorable thereto. But there is obviously a limit to what alterations in the fluid may be

[8] Gorke, H., *Deutsch. med. Woch.*, 1922, xlviii, 1166.

expected to accomplish. The stones were situate at and about the constriction in a cannula of not more than 2 mm. greatest diameter, and with rigid walls. It is regrettable that no quantitative observations were made on the carbonates of the bile, of which, as already remarked, the calculi principally consisted.

Comparison of Hepatic and Renal Obstruction.

For any proper comparison of the effects of biliary obstruction with those of obstruction to the ducts of another gland, the complicating activities of the gall bladder must be ruled from account. For the *receptaculum bilis* is much more than its name implies, being an organ of highly specialized functions and quite as unrelated to the secretory system of the liver as the urinary bladder is to that of the kidney. The comparison with the kidney will be extended further, since of all the glands with ducts it has been most studied. There are available numerous data upon the effects of a pressure obstacle to secretion by the renal tissue.[8]

Both the liver and the kidney go on secreting after the ducts from them have been entirely closed; but as soon as the pressure within the distended ducts has risen to a certain point, the product of their activity is turned back into the organism. The stretching of the tissues incidental to the obstruction is far more marked in the case of the kidney, as would naturally follow from the circumstance that urine does not cease to be secreted into the proper channels until a high pressure has been attained,—50 to 70 mm. of mercury in the case of the dog.[9] There results the hydronephrotic sac. Bile ceases to be secreted when the pressure in the ducts of the dog reaches 350 mm. of the fluid itself,[2] or in other words, when the resistance is only one-third to one-half that required to bring the flow of urine to a standstill. The difference is sufficient to account for the absence of a spectacular bagging-out of the liver, irrespective of any special resistance on the part of the hepatic tissue. In human beings small cystic dilatations on the surface of the liver have occasionally been noted.[8] The urine at first secreted above an obstruction is gradually replaced by a fluid devoid of the typical characters of the secretion.

[9] Cushny, A. R., The secretion of the urine, London, New York, Bombay, Calcutta, and Madras, 1917, 89.

Kaufmann[10] has likened it to the fluid of *hydrops vesicæ felleæ*, of a gall bladder pathologically altered. So too the bile at first pent in obstructed ducts is replaced by a non-characteristic "white bile," in the absence of the normal gall bladder activity for concentration. Kausch[11] employs the expression hydrops of the biliary system in this connection. When the pressure obstacle to urinary secretion does not wholly prevent it, the output of certain substances is diminished, both as a whole, and per cubic centimeter, just as happens with cholesterol and bilirubin on partial biliary obstruction.[9] Whether the concentration of certain biliary constituents is increased, as is that of urea,[9] cannot at this writing be stated.

In any comparison of the renal and hepatic changes after obstruction it is necessary to keep in mind that the kidney tissue is lodged in a pair of organs and the hepatic in but one. Yet the differences thus entailed are not so considerable as one might suppose. The bile radicles draining into one branch of the hepatic duct are so little connected with those to another[12] that the liver mass may be thought of as consisting of a number of separate biliary units within a single capsule, like separate watershed systems eventually tributary to a single river. Obstruction to the duct from one of these systems may in many respects be likened to obstruction of a single kidney. The ensuing changes are strikingly similar.

The pressure under which the fluid is held on urinary obstruction is such as to bring about a compression of the renal veins with serious circulatory disturbance. Similarly when the bile ducts from a part of the liver are stopped, the portal flow through the region is impeded and diverted.[13] As result of the circulatory changes in the kidney[14] the organ, after passing through a hydronephrotic stage, becomes atrophic and shrunken.[15] So too a complete parenchymal atrophy,[16]

[10] Kaufmann, E., in Riemer, G., Lehrbuch der speziellen pathologischen Anatomie für Studierende und Ärzte, Berlin, 4th edition, 1907, 836.

[11] Kausch, W., *Mitt. Grenzgeb. Med. u. Chir.*, 1911, xxiii, 138.

[12] McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1921, xxxiii, 731.

[13] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxii, 249.

[14] Kornitzer, E., *Z. urol. Chir.*, 1922, ix, 165.

[15] Aschoff, L., in Aschoff, L., Pathologische Anatomie, Jena, 4th edition, 1919, ii, 552.

[16] Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

and from a like cause,[17] follows on local biliary obstruction. A progressive connective tissue overgrowth accompanies the atrophy in both kidney and liver. The causes for it need not be gone into. When hepatic or renal obstruction affects all of the gland, permitting no escape whatever of secretion, death ensues, before the tissue changes just enumerated have progressed far.

Stasis, distention, vascular disturbance, sclerosis, and parenchymal atrophy,—these are common not merely to the liver and kidney but to all glands of which the ducts have been obstructed. It is safe to say that the underlying physiological pathology is much the same for all. And yet the essential identity of the liver changes with those occurring in other glands seems not to have been recognized in the past. A principal reason is to be found in the activities of the gall bladder to cloud the issue in a literal sense by thickening and concentrating the bile retained above an obstruction. As result of this, and of infection, and of differences in the agents determining biliary obstruction, the conditions found at autopsy or operation in human beings have an appearance of great diversity. Of necessity each instance has been considered by itself; and the lack of a comprehensive term to join one with another and to cover the general state of affairs has not been felt. In the present paper and those preceding it the nature and relative simplicity of the physiological factors responsible for the liver findings have been brought out. With this approach to an understanding of the physiology of biliary obstruction there would seem to be some usefulness in a word designating its results. We suggest that *hydrohepatosis* would be no misnomer, and that the term may be helpful as indicating the likeness of the liver changes to gland changes in general after duct obstruction, and to those of the kidney in particular. When, through gall bladder activity, the duct content above an obstruction is permanently thickened and pigmented, the hydrohepatosis is *concealed*; but in the absence of this disturbing influence, when the duct system is filled with a clear, watery fluid, the condition is a *manifest hydrohepatosis*.

[17] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609.

SUMMARY.

In bile that is secreted against an abnormally high pressure, as during partial obstruction, the pigment, cholate, and cholesterol outputs are all cut down, and so much more than is the fluid bulk that the concentration of the substances per cubic centimeter of bile is notably lessened. The fluid obtained at the greatest pressure compatible with secretion contains traces only of the typical biliary constituents. The bearing of these alterations in the bile on the consequences of partial biliary obstruction is discussed.

An analysis of the liver changes following biliary obstruction brings out their essential likeness to the changes that occur under similar circumstances in glands in general and the kidney in particular. The major physiological factors concerned in the development of hydronephrosis and in the liver changes after biliary obstruction are identical. We would suggest that the term *hydrohepatosis* as applied to the liver condition would be useful not merely to designate it but to indicate the principles underlying its development. In clinical instances of biliary obstruction, the likeness to hydronephrosis is often hidden because of the activity of the gall bladder to render the stasis bile dark and thick. There is then a *concealed hydrohepatosis*, differing merely by the character of the duct content, from the *manifest hydrohepatosis* with "white bile," that is found when the gall bladder fails to act.

STUDIES ON THE TOTAL BILE.

IV. The Enterohepatic Circulation of Bile Pigment.

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According to accepted opinion the pigment of the bile represents waste from the hemoglobin molecule, and as such is eliminated from the organism. Yet the belief is well-nigh general that this waste, after undergoing a change to urobilin, is reabsorbed in part from the intestine. Why should the reabsorption take place? Does it indeed occur? Is any unchanged bile pigment reabsorbed? These are questions which have led to much work and more speculation. The present paper is concerned with but one of them, namely with the reabsorption of bile pigment, irrespective of its form.

Whipple,[1] the most recent investigator of the theme, states that: "There is no evidence that bile pigment or stercobilin is absorbed from the intestine." And yet there are phenomena on record which might be construed as pointing to such an occurrence. It is true that Hooper and Whipple could find no indication in the bilirubin yield from fistula dogs that any pigment from bile given by mouth was absorbed.[2] But they dealt with the yield of 8 hours only in each 24. Baldi[3] noted as far back as 1883 that following the administration to fistula dogs of beef bile green with biliverdin, the dog bile changed from brown to green; and Schiff observed that the urine of animals so treated gave Gmelin's reaction. Stadelmann[4] presents figures which show that when his fistula dogs were prevented from licking up the bile secreted during 12 hours of each 24 the output of pigment during the remaining period lessened somewhat, though not greatly. Since Wertheimer's work[5] there has been no doubt of the fact that the normal

[1] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

[2] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916–17, xlii, 264.

[3] Baldi, D., *Arch. ital. biol.*, 1883, iii, 389.

[4] Stadelmann, E., *Z. Biol.*, 1896, xxxiv, 1.

[5] Wertheimer, E., *Arch. physiol. norm. et path.*, 1891, iii, series 5, 724; 1892, iv, series 5, 577.

liver will rapidly remove bile pigment from the blood stream and resecrete it into the bile.

Our experiments were carried out with dogs permanently intubated for the collection of the total bile.[6] The methods employed in the care of the animals and the quantitation of the bile constituents have already been described.[7] Often the secretion remained sterile during many weeks of collection, until the animal was killed. Such infections as occurred were frequently limited to the collecting apparatus; and the organisms were always non-pathogenic, and failed to effect quantitative changes in the bile pigment, as proven by comparing specimens of the infected bile incubated *in vitro* with others kept in the ice box, and by similar tests with biles previously sterile, and now infected in the test-tube with the organisms. The specimens compared were treated with acid alcohol according to Hooper and Whipple's method, and viewed in the Duboscq colorimeter.

The observations of Baldi received an indirect confirmation early in the work. When our dogs were fed cooked liver of the sheep or ox, the bile for several days thereafter was noted to be green instead of the previous yellow-brown. But as Wertheimer remarks of Baldi's finding, such a change may be the result merely of an oxidation of the bilirubin of the dog bile itself to biliverdin, and in any event it is essentially non-specific. Whenever air was present in the collecting balloons we employed there occurred some conversion of bilirubin to biliverdin. But this source of error was avoidable. Perhaps our best evidence for a significant relationship between liver feeding and the color change is to be found in the fact that the association of the two attracted our attention prior to knowledge of Baldi's report.

Wertheimer tested for an enterohepatic circulation of pigment by administering sheep bile to dogs and searching the dog bile afterwards for cholohematin, present in the bile of the sheep and recognizable by spectroscopic means. He made collections directly from the common duct of etherized animals and had no difficulty in finding cholohematin after the injection of sheep bile into the circulation. But his experiments to determine a resorption of it from the intestine

[6] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

[7] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

during the few hours time that the conditions permitted yielded negative results. Our own endeavors, with unanesthetized and vigorous dogs given sheep bile by mouth, have been more successful.

Experiment 1.—A large number of unemptied gall bladders of the sheep were obtained from a slaughter house. The bile in some was green and in others a pale yellow. These latter were discarded and the green secretion was pooled. When given as such in quantity to dogs it nearly always induced vomiting, but when somewhat concentrated on the water bath it proved tolerable. Chloroform extracts of the material, evaporated and dissolved in ether, according to the method of MacMunn,[8] showed with the spectroscope the four bands characteristic of cholohematin, namely one in the red about the line C, well marked and broad, another in the yellow near D, and two in the green. Portions of the material were fed by stomach tube to two dogs, the bile of which in chloroform extract had been spectroscopically examined for some days previously.

Dog 19, a male bull terrier weighing 13 kilos, yielded on the 37th to 40th days after intubation a bile the extracts of which showed with the spectroscope a narrow, pale band in the yellow-green as well as the usual obscuration of both ends of the spectrum. Early in the 41st day of period of collection two attempts were made to feed sheep bile, as such, by stomach tube. The entire quantity was vomited practically at once, and the spectrum for the period did not differ from that of the preceding days. Early in the 42nd period 35 cc. of sheep bile concentrated from an original bulk of 93 cc. was given by tube, and slightly more than an hour later 40 cc., representing 100 cc. of the secretion. Practically all was retained despite some retching. Extracts of the dog bile for the 24 hour period showed plainly the spectrum characteristic of cholohematin. It was much stronger in the next specimen of the 43rd collecting period, was still well defined in that of the 44th, and gradually disappeared thereafter, with no trace in the 47th specimen.

Dog 25, rough haired terrier of 15½ kilos. Extracts of the bile obtained from this animal, during the preliminary observations of the 36th to 40th collecting periods inclusive, yielded the spectrum already described for the control specimens from Dog 19. At the beginning of the 41st period of collection 30 cc. of concentrated sheep bile, corresponding to 80 cc. of the original material, was given by tube, and followed 75 minutes later by 45 cc. more, equalling 120 cc. of the unchanged secretion. The same material was used as in the case of Dog 19, and just as in this animal the spectrum of cholohematin appeared and persisted for 5 days.

We have had abundant opportunity to confirm Wertheimer's statement that, under ordinary conditions, the bile of fistula dogs does

[8] MacMunn, C. A., *J. Physiol.*, 1885, vi, 32.

not yield a spectrum which can be confused with that of cholohematin. The temporary appearance of the spectrum of the pigment after feeding it to dogs, in the form of sheep bile, would seem to prove conclusively that it is absorbed from the intestinal tract and resecreted by the liver.

The sheep bile used was lightly pigmented with biliverdin. Following its administration there was frequently a change in the color of the dog bile from yellow to green, as after feedings of the liver tissue of the herbivora, although the total pigment content of the dog bile underwent no appreciable alteration. But extremely little biliverdin is required to render bile green.

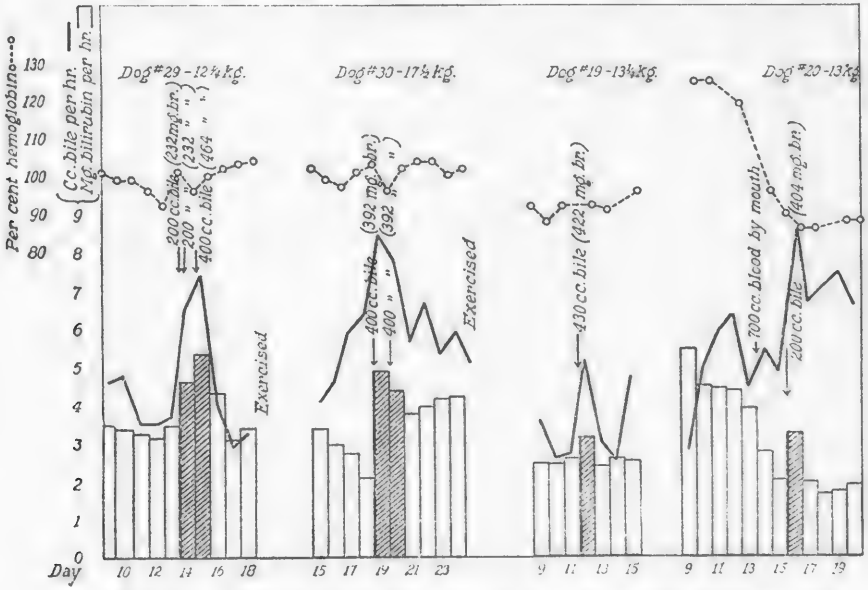
Experiments were now begun to test whether the giving of bilirubin by mouth in the form of dog bile would cause a rise in the output of bilirubin by the liver.

Since bilirubin is altered by weak acids, and in this instance would be submitted to the acid of the gastric juice, it seemed best to give large quantities in order to provide opportunities for some at least to reach the intestine in unchanged form. The material consisted of pooled 24 hour specimens derived, not infrequently, from the animal to be tested. The precise amount of bilirubin so administered was determined by the same method employed to quantitate the daily yield from the animal.[7] The bile was given by stomach tube at the beginning of a collecting period, with, sometimes, a second portion several hours later. It was well tolerated. All the dogs were in good condition except one (No. 23). Shortly after the initial gavage they were fed as usual. The character of the diet, which was always poor in fat, and the amount taken were recorded, but the data have no importance in the present connection, since, as has been shown in a previous paper, diets such as we employed are void of effect on the yield of bilirubin from day to day. Nevertheless, a standard ration was fed throughout the experimental and control periods, save in special instances. The hemoglobin percentage of the blood was closely followed, because marked variations in it are usually accompanied by similar variations in the bilirubin output.[7]

The extreme cholorrhea induced by bile feedings was occasionally associated with a curious color change in the pigment when submitted to acid alcohol prior to readings in the colorimeter. The ordinary change is through a transient green to a more enduring blue-green with which latter hue the inorganic standard corresponds. But in a few of our specimens the abnormally profuse bile turned instead to a brilliant purple. Urobilin was never present. The bile fed gave always the ordinary color reaction. We have had occasion to note that normal gall bladder bile,—which is sterile,—frequently changes under the influence of

acid alcohol to a purple hue, possibly from the presence in it of bilicyanin.[9] Whatever the cause for the color peculiarity it renders the quantitation of pigment difficult. Occasionally this could be carried out only by a comparison of water-diluted specimens with similarly treated samples of bile of known pigment content.

The charts present the essential data on bile quantity and bilirubin yield for the entire series of thirteen experiments (Text-figs. 1 to 5).


Per cent hemoglobine
Cc. bile per hr.
Mg. bilirubin per hr.
Dog #29 - 12¼ kg.
Dog #30 - 17½ kg.
Dog #19 - 13¾ Kg.
Dog #20 - 13 Kg.
200 cc. bile (232 mg. bir.)
200 " " (232 " ")
400 cc. bile (464 " ")
Exercised
400 cc. bile (392 mg. bir.)
400 " " (392 " ")
Exercised
430 cc. bile (422 mg. bir.)
700 cc. blood by mouth
200 cc. bile (404 mg. bir.)
Day

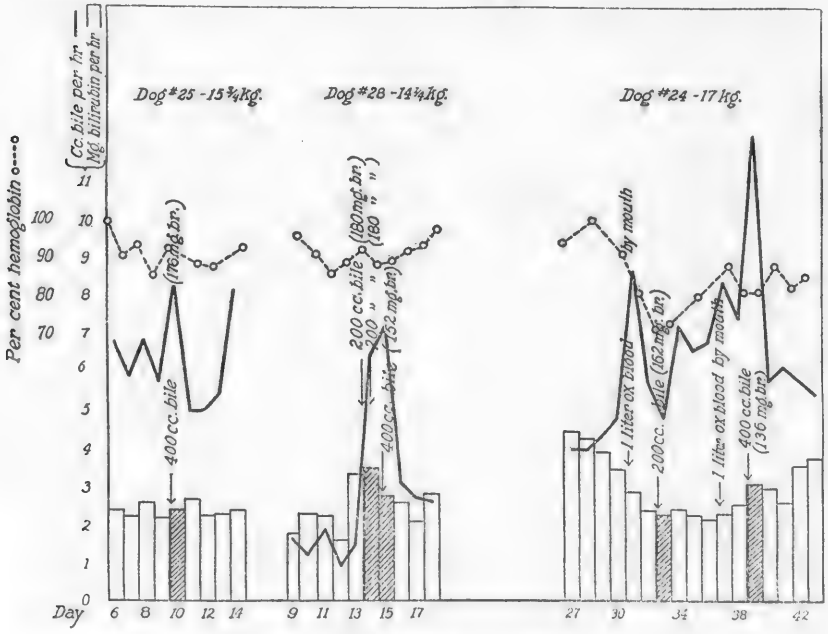
TEXT-FIG. 1. Effects of bile feedings. (In Dog 30 each bile feeding was followed by diarrhea.)

In six of the ten experiments in which bile was fed on a single day or on two successive ones (Text-figs. 1 and 5) there occurred a marked rise in the bilirubin output followed by nearly as abrupt a drop to the previous level. The changes were too great and abrupt to be referable to intercurrent variations. In the remaining four instances (Text-fig. 2) the pigment output either underwent no alteration or the increase was insignificant. In one of them (Dog 24), as in one of the series giving positive results (Dog 20), the hemoglobin percentage of

[9] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

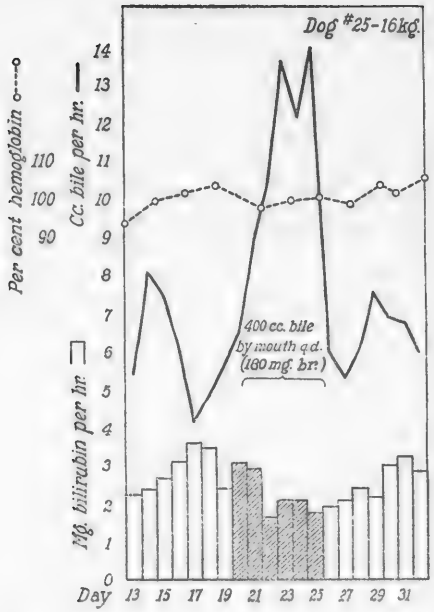
the blood varied considerably during the observations, and the bilirubin output underwent corresponding changes.

In three animals bile was administered by stomach tube on each of 6 to 10 successive days. In one such instance the bilirubin output altered but little (Text-fig. 3); in another it underwent occasional increases, more abrupt, and one of them far greater, than any observed

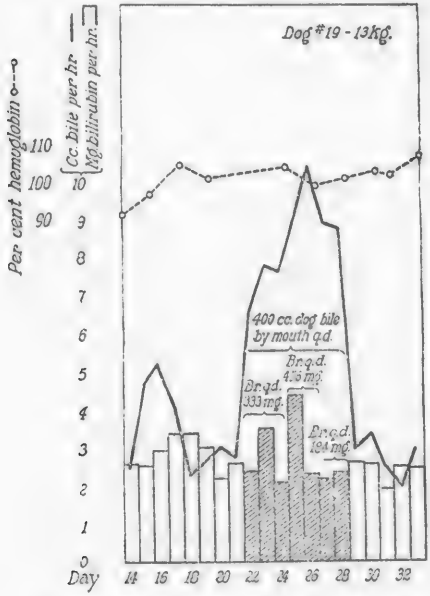

Cc. bile per hr.
Mg. bilirubin per hr.
Per cent hemoglobin
Dog #25 - 15¾ Kg.
Dog #28 - 14¼ Kg.
Dog #24 - 17 Kg.
400 cc. bile
(116 mg. bilirubin)
200 cc. bile (180 mg. bilirubin)
200 " " (180 " ")
400 cc. bile (152 mg. bilirubin)
by mouth
1 liter ox blood
200 cc. bile (162 mg. bilirubin)
1 liter ox blood by mouth
400 cc. bile (136 mg. bilirubin)
Day

TEXT-FIG. 2. Effects of bile feedings.

during control periods (Text-fig. 4); while in a third (Text-fig. 5) the pigment yield doubled in quantity almost immediately that the feedings were begun, and increased till toward the end of them it had more than quadrupled. This dog (No. 23) had long been ailing, owing, as autopsy showed, to a high intestinal obstruction; and the administration of the bile was followed by a remarkable change for the better in its condition. The protocol has such interest that it will be summarized.

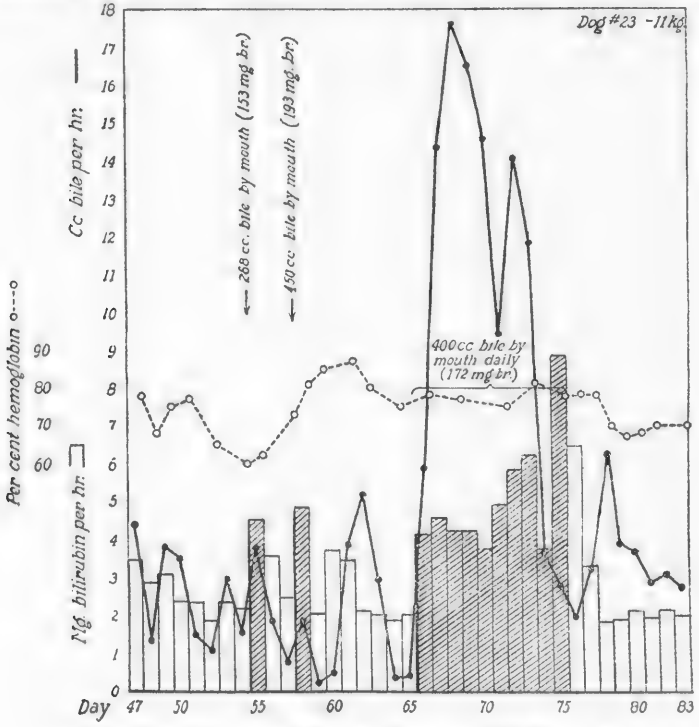


TEXT-FIG. 3. Effects of repeated bile feedings.



TEXT-FIG. 4. Effects of repeated bile feedings.

Dog 23, a brown spaniel, was found pregnant at the operation to insert a bile-collecting apparatus, and a hysterectomy was done. Immediately afterwards the animal weighed 15¾ kilos. For some weeks it remained in good condition, save for a gradually developing secondary anemia of greater severity than that ordinarily following total loss of the bile. There was loss of weight, and a slight purulent discharge where the collecting tube emerged from the abdomen. The bile itself remained sterile until just before the animal was killed, after nearly 3 months of observation. 6 weeks from the time of the operation the animal



TEXT-FIG. 5. Effects of single and repeated bile feedings.

was sluggish and its appetite was noted to be capricious. It now fell ill of a severe, intercurrent bronchitis that greatly depleted it. After 65 days in all, when it had recovered, it weighed only 11 kilos, ate almost nothing, and lay languidly in its cage. A variety of diets poor in fat failed to tempt it, and death seemed imminent. In two successive 24 hour periods only 9.1 and 9.8 cc. of bile respectively was secreted, instead of the 125 to 240 cc. given earlier. Yet

these small portions held the usual 24 hour quota of bilirubin. Bile feedings were now begun, of 200 cc. by gavage twice each day. Within 3 days the animal was lively, eating food of a sort previouly refused, and gaining in weight. But after bile had been administered in large quantity for 7 days and the condition of the animal had become good, a watery, bile-stained diarrhea developed, accompanied by anorexia, and by the 10th and last day of bile feeding these disturbances had become severe. Immediately that the feedings were stopped the diarrhea ceased, the appetite returned, and during the next few days the dog appeared better than for some weeks. But soon pronounced symptoms of intestinal obstruction manifested themselves, rendering it necessary to sacrifice the animal,—on the 85th day. Autopsy showed that a loop of small bowel had become included in an adhesion near the exit wound for the collecting tube, with result in practically complete obstruction about 4 feet beyond the pylorus. Above this point the intestine was greatly distended, and full of a clear, nearly colorless fluid, while below it was empty. The liver and bile ducts were normal.

DISCUSSION.

The evidence here presented for the absorption of bile pigment from the intestine is of unequal value. The change in color of dog bile after the feeding of the green bile or the liver tissue of herbivora counts as no more than suggestive in this connection. The appearance in the bile of cholohematin, after administration of the pigment by mouth, shows conclusively that a substance nearly related to bilirubin undergoes enteral absorption and excretion by the liver. But as a matter of fact, recently proven, this is shown sufficiently by the presence of the substance in the bile of sheep and cattle. For cholohematin, or bilipurpurin, as it has also been called, is no true constituent of the bile of the herbivora, such as MacMunn[8] and Wertheimer[5] supposed it to be, but is of extrinsic origin, being a derivative of the chlorophyl of the food and identical with phylloerythrin.[10] Only when the fodder contains chlorophyl is it found in the bile.[11]

The results of the feeding of dog bile leave no doubt that the bilirubin output was increased thereby in many instances. But why was it not increased in all? One reason is that circumstances were frequently unfavorable to absorption from the intestinal tract. For bile in quantity by mouth often acts as a purgative and in consequence may itself be so rapidly hurried through the bowel as to appear unchanged

[10] Marchlewski, L., *Z. physiol. Chem.*, 1904–05, xliii, 464.
[11] Marchlewski, L., *Z. physiol. Chem.*, 1905, xlv, 466.

in the stools. This took place in several of our animals; but unfortunately it did not attract attention until late in the work, so that the protocols cover the occurrence insufficiently. In contrast to such instances is that of Dog 23 in which the fed bile was probably retained longer than usual in the upper part of the intestinal tract, owing to partial intestinal obstruction. The enormous increase in the output of bilirubin may in this way be explained.

Is the pigment increase necessarily to be attributed to intestinal resorption? It is certainly not due to flushing out by the quickened bile stream. The independence of the rates of bile flow and of bilirubin secretion has impressed practically all workers; and we have recently recorded some striking instances illustrating it. One is mentioned in the history of Dog 23 here given. Furthermore, a flushing out of pigment could scarcely endure for days; and in any case there should follow a drop in the bilirubin output below the previous level until the substance has again accumulated in the organism. No such drop was to be seen.

A relatively small liberation of hemoglobin by blood destruction would account for the pigment increase. It was in this way that Stadelmann explained such increase as he observed after the feeding of large quantities of bile salts to fistula dogs. He fed no greater amounts than our dogs may have received in the form of bile, so his view deserves attentive consideration in connection with the present findings. If it be assumed that 1 gm. of bilirubin in the bile represents approximately 1 gm. of hematin from blood,[12] that the hemoglobin molecule yields 4 per cent of hematin by weight, and that 100 cc. of dog blood with 100 per cent of hemoglobin by our Newcomer standard contains 13.8 gm. of hemoglobin,—as special determinations showed to be the fact,—then the destruction of but a few extra cubic centimeters of blood would provide for the added output of bilirubin after bile feedings. In Dog 29, for example, only 0.105 gm. of additional pigment was eliminated in the 3 days following the feedings. The breaking down of 19 cc. of blood with 100 per cent of hemoglobin, such as was possessed by the animal, would account for this. So small a destruction need bring about no appreciable change in the

[12] Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin and Vienna, 4th edition, 1920, i, 746.

hemoglobin per cent of a dog weighing 12 kilos. In the extreme instance of Dog 23 an average of approximately 0.072 gm. of extra pigment appeared in the bile on each of 11 successive days, which an extra destruction daily of 16.3 cc. of blood with 80 per cent hemoglobin would suffice to explain. The state of the animal was so abnormal that the loss might have occurred without evidence of it in the routine blood examination.

As against a destruction of corpuscles by cholates, the remarkable properties of the plasma to prevent hemolysis by such salts[13] should be recalled. This had not been recognized at the time when Stadelmann worked. In an experiment described by Sellards[14] the presence of blood serum in a 1 to 30 dilution sufficed to protect a few red cells from the hemolyzing influence of 1 mg. of sodium taurocholate, the salt of dog bile, in a total fluid mixture of 1.5 cc. It seems scarcely possible that under the conditions of intestinal absorption any comparable concentrations of taurocholate could have occurred. In 24 hour specimens from dogs given large amounts of the cholate of dog bile, Wisner and Whipple found no increase of pigment[15] nor was one observed by Hooper[16] in 6 hour specimens. Stadelmann studied the bile of 12 hours in each 24. It is possible that the pigment increases which he observed were not the result of a greater total output of the substance but merely of recurring temporary alterations in the rate of elimination, such as follow the administration of carbohydrates.[17]

As the case stands at present there is every reason to believe that the increased bilirubin output after bile feeding is the result of an enterohepatic circulation. Why indeed should not bilirubin be reabsorbed and resecreted if this happens with cholohematin, as is assuredly the case?

The proportion of bilirubin presumably reabsorbed in our experiments was sometimes negligible, and again great. In Dog 23 it

[13] Lüdke, H., *Centr. Bakt., 1te Abt., Orig.*, 1906, xlii, 455, 552. Bayer, G., *Biochem. Z.*, 1908, xiii, 215.

[14] Sellards, A. W., *J. Exp. Med.*, 1909, xi, 786.

[15] Wisner, F. P., and Whipple, G. H., *Am. J. Physiol.*, 1922, lx, 119.

[16] Hooper, C. W., *Am. J. Physiol.*, 1916–17, xlii, 280.

[17] Rous, P., Broun, G. O., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 421.

amounted, as above mentioned, to 72 mg. of the 172 mg. administered each day, or 42 per cent. The giving of bile in bulk on one or two occasions would seem far less favorable to reabsorption than the intermittent delivery into the intestine of smaller quantities, as happens under normal circumstances. Quite possibly a part of the pigment in duodenal specimens obtained from human beings is representative, not of a final elimination of the substance by the organism but of an enterohepatic circulation of it. A great deal of bilirubin might thus circulate under pathological conditions.

That bile feeding is of benefit to man and other animals losing the secretion by a fistula has long been acknowledged. But many acknowledged principles are not sufficiently acted upon. According to a qualified authority, "Human beings who are losing all their bile through a fistula not only have no appetite but can hardly be forced to take food, and emaciate very rapidly. Intensely jaundiced patients may live years, but if, following an operation upon one of these cases, all the bile escapes by the fistula the loss of weight is very rapid and death may soon result if the patient is old."[18] It is in this general relationship that the astonishing recovery of Dog 23 on bile feeding has seemed worth recording.

SUMMARY.

In dogs fed the green bile or the liver tissue of herbivora, the bile later secreted frequently becomes green, changing from the previous yellow-brown. When they are fed sheep bile that contains cholohematin, their bile comes to contain this pigment. When they are fed dog bile in quantity a well marked increase in the output of bilirubin by the liver frequently follows. Taken together, these facts indicate the existence of an enterohepatic circulation of bile pigment.

[18] Extract from a letter to one of the authors by Dr. W. S. Halsted.

BOTULISM.

STUDIES ON THE MANNER IN WHICH THE TOXIN OF CLOSTRIDIUM BOTULINUM ACTS UPON THE BODY.

I. The Effect upon the Autonomic Nervous System.*†

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The generally accepted conception of the symptom-complex of *botulinus* intoxication in human beings is best summarized by Van Ermengem (1) who wrote:

> The botulism syndrome consists essentially in a group of neuroparalytic phenomena: disturbances of secretion of the gastrointestinal tract and symmetrical motor paralysis, complete or partial, probably caused by lesions of the medulla oblongata, of the pons, especially of the nuclei of the various cerebral nerves, and of the anterior horns of the spinal cord. It is characterized by an inhibition of, or increase in the secretion of saliva and of the buccopharyngeal mucus, by more or less complete external and internal ophthalmoplegia (blepharoptosis, mydriasis, paralysis of accommodation, diplopia, and internal strabismus); by dysphagia, aphonia, obstinate constipation, and retention of urine; by general weakness of contraction in all the voluntary muscles; by an absence of fever and of disturbance of the general sensation or of intelligence.

In 1894–96 Van Ermengem proved that the immediate cause of botulism in human beings or in animals is a toxin which is elaborated by the growth of *Clostridium botulinum* in preserved foods, and since that time there have been many attempts to ascertain the manner in which the toxin acts upon the body to produce these various signs and symptoms. The majority of investigators have

* These experiments are a part of an investigation of botulism which was made in California by the United States Public Health Service, Stanford University, and the University of California under a grant from the National Canners' Association, the Canners' League of California, and the California Olive Association.

† The experiments were performed in the Laboratory of Experimental Medicine at Stanford University Medical School and in the Department of Physiology at Stanford University. We desire to express our indebtedness to Professor E. G. Martin for the facilities of his department and for his interest and advice throughout the course of the investigation.

followed the lead suggested by Van Ermengem by attempting to demonstrate organic changes in the cells of the nuclei of the cranial motor nerves which would afford anatomical cause for the characteristic disturbances of function.

Marinesco (2) was the first to study the tissues of the nervous system in this way, his observations being made upon the brains and cords of animals in which Van Ermengem had induced experimental botulism. He described changes in the finer structure of the ganglion cells of the brain and cord. They were most marked in the cord in the enlargements which correspond to the extremities, less marked in the medulla oblongata, pons, and peduncles, and almost absent in the ganglion cells of the cerebral hemispheres.

He observed the changes in the nuclei of the nervus hypoglossus, the nucleus ambiguus, the dorsal nuclei of the vagi, the Purkinje cells of the cerebellum, and the middle nucleus of the nervus oculomotorius, and described them as follows:

> In the first stage one finds only a diminution of the chromatophilic elements extending from the periphery of the cells, but later the Nissl granules are reduced to fine powder-like granules. Still later there is absorption of the achromatophilic substance and vacuoles appear, and in the vicinity of these lesions there is proliferation of the glia cells to produce neuronophages which take up the destroyed material.

He did not find any destruction of the nerve fibers.

Kempner and Pollack (3) reported similar destructive lesions in the nerve cells of the brain and cord but also found an earlier stage in the protoplasmic degeneration which they described as a "*klumpige Schwellung*" of the cell granules. They did not observe any proliferation of the glia cells. These authors noted that when an animal was protected from the toxin by the administration of *botulinus* antitoxin, the lesions in the nerve cells persisted for at least 2 weeks after the clinical manifestations had cleared up, and they were unable to demonstrate any correlation between the nerve cell damage and the severity of symptoms.

Ossipoff (4) agreed with Marinesco that the lesions in the cells were most marked in the cord, especially in the portions which supplied the nerves to the extremities, and that they were less marked in the bulb, but he also found similar lesions in the Purkinje cells of the whole brain. He stated, however, that he had not found sufficient reason to assume that the changes in the nerve cells are specific of botulism and different from changes which occur after the administration of other toxins such as diphtheria and tetanus toxins.

Römer and Stein (5) made careful studies of the nuclei of the oculomotor nerves in monkeys which they had injected with *botulinus* toxin and they found tigrolysis, pycnosis, clumping of Nissl granules, and, in more advanced cases, complete disintegration of cellular structure in the impaired median nucleus. They concluded that there was sufficient histologic change in the nerve cells to account for the disturbance of function in the muscles of the eyes of these animals.

All these observers based their conclusions upon a study of the tissues of animals in which the intoxication had been experimentally produced, and there are few reports in which analogous studies have been made of the brains of

human victims of botulism. Bürger (6) recorded that the cells of the cerebrum were normal in the brain of a woman who died on the 16th day of the intoxication but that there was pulverization of the Nissl granules and eccentric displacement of the cell nuclei in the region of the nuclei of the oculomotor nerves. Dorendorf (7) described the central nervous system of a patient who died 15 days after the onset of bulbar paralysis, and 17 days after ingesting the *botulinus* poison. The ganglion cells in the spinal cord and cerebellum were intact but that there were marked chromatolytic changes in the medulla and pons and in the region of the corpora quadrigemina. Marked chromatolysis and cell destruction in the nuclei of the vagus, oculomotor, and abducens nerves were observed and in the pons there was scarcely an intact cell to be seen. There was little change in the cells of the hypoglossus nucleus and in the ganglion cells of the cerebrum.

A more recent study of the central nervous system of human victims of botulism is that of Semerau and Noack (8) who described two cases. They observed "more or less marked" changes in almost all the motor nuclei of the cranial nerves and in the tracts leading from them. These changes were especially marked in the cells of the nuclei of the vagus, glossopharyngeal, and hypoglossal nerves, and less marked in those of the facial nerves and of the nerves supplying the eyes. They reported the changes to be relatively more severe in the cells of the anterior horn of the spinal cord, particularly in the cervical region, and noted that they decreased in severity as the lumbar portion of the cord was approached.

These authors recognized the degeneration in the nerve cells by a diffuse cloudiness of the stained cell body due to blackening of the lipochrome pigments with osmic acid, by powdery destruction of the Nissl granules, and by eccentric displacement of the cell nucleus. There was shrinkage, irregularity, and fraying of the cell contour in many instances. The motor fiber tracts of the brain stem and upper part of the spinal cord were also involved but no evidence of inflammation in the meninges was found. The conclusion was drawn that the observations had proved that in human victims of botulism there is correlation between the symptomatology of the intoxication and organic changes in the brain and spinal cord.

The published reports of studies of the finer structure of the nerve cells in victims of *botulinus* intoxication in the United States do not agree with those recorded in the German literature. Ophüls (9) made a careful study of the brain of one human victim of botulism in which "the nuclei studied were those of the oculomotorius (third), trochlearis (fourth), trigeminis (fifth), abducens (sixth), facialis (seventh), acusticus (eight), glossopharyngeus (ninth), vagus (tenth), and hypoglossus (twelfth) nerves." He found that: "The Nissl granules stain unusually well in all sections, especially in the large motor ganglion cells. They are quite normal in size and arrangement. Nothing unusual could be detected in spite of especially careful study of them. The nuclei of the ganglion cells are perfectly normal."

One of us (10) has had the opportunity of examining sections from the tissues of eleven other human victims of botulism which have occurred in the United States, and of a large number of animals in which the intoxication was experimentally produced. In a few of the animals, especially in cats, there was some evidence of change in the nerve cells of the motor nuclei; the cells were shrunken, the contour was irregular, the protoplasm was opaque and occasionally vacuolated, the nuclei stained poorly, and at times the whole structure of the cell was lost and there remained only a hyaline mass in the position it had occupied. But in the great majority of animals and in all the human cases in which the tissues were sufficiently well preserved to permit of study, there was no indication of the changes which have been described by the European authors, and in a series of experiments in which the vital staining methods of Kiyono (11) and Goldmann (12) were employed, there was no indication of pigment granules in any of the nerve cells such as were described by MacCurdy and Evans (13) in the damaged ganglion cells in poliomyelitis. In fact, nothing was found which could be interpreted as a probable central cause for the typical signs and symptoms of *botulinus* intoxication.

Analysis of the reports of the European authors shows that although they all approached the problem from the same angle there is no complete accord either in their observations or in the interpretations which they placed upon their findings, and it is difficult to correlate their findings with the disturbances which are observed in a clinical case of botulism. For instance, although among the earliest and most constant signs of *botulinus* intoxication are blepharoptosis, diplopia, mydriasis, and loss of accommodation for near vision, all disturbances which are caused by imperfect function on the part of the oculomotor nerves, Marinesco (2), Ossipoff (4), and Semerau and Noack (8) recorded that the lesions were relatively least numerous and severe in the region of the nuclei of the third nerves. Römer and Stein (5) were the only ones who believed that they had demonstrated sufficient lesions in the cells of the oculomotor nuclei to account for the clinical manifestations which were observed. And again, although all clinical observers agree that while there is marked general muscular weakness in botulism there is rarely any true paralysis of the muscles of the extremities, almost all the European observers noted that the lesions are much more numerous in the nerve cells of the upper portion of the cord than in the basilar nuclei in the brain, and Semerau and Noack (8) stated that the chief site of lesion is in the cells of the anterior horns of that part of the cord which supplies the upper extremities.

If the disturbances in function which are characteristic of *botulinus* intoxication are dependent upon visible lesions in the ganglion cells from which the various organs receive their enervation, it is inconceivable that there should be no paralysis of the organs which receive their nerve supply from ganglion cells which show the greatest change or that there should be the most constant and severe disturbances in those whose cells are less severely damaged; and, moreover, if the disturbances in function are dependent upon organic changes in the nerve cells, one cannot understand how function can be wholly reestablished in the victims who do not succumb to the poisoning notwithstanding the fact that, as Kempner and Pollack (3) noted, the lesions may remain demonstrable in the nerve cells of animals for at least 2 weeks after all the clinical manifestations of the intoxication have entirely cleared up.

The only conclusion that can be drawn is that the method of histologic study has not established the facts as to how the *botulinus* toxin acts upon the body and that other methods of investigation should be employed. It is the purpose of this and of a later report to discuss the results of a series of experiments in which the disturbances of function in *botulinus* intoxication were studied by methods of experimental physiology and to attempt through them to explain the way in which the toxin acts upon the body.

The method of approaching the problem was suggested by the discussion of Gaskell (14) as to the significance of Dale's observations (15) upon the action of acetyl choline because of the striking fact that toxic doses of acetyl choline appear to stimulate the portions of the nervous system which are depressed in *botulinus* intoxication. The first series of experiments, therefore, was planned to obtain a number of quantitative physiological observations concerning the conduction of nerve impulses along the various nerves of the autonomic system in animals which had been poisoned with *botulinus* toxin for comparison with similar data obtained from normal animals, but it eventually became necessary to enlarge the scope of the investigation to include comparative studies of the function and rate of the onset of fatigue of the skeletal muscles of normal and of botulism animals.

The results of the experiments which deal with the effect of the toxin upon the autonomic nervous system are discussed below, those

which have to do with the effect upon the nerve supply of the skeletal muscles will be described in a later report.

In order to obtain comparative data we employed the method of quantitative faradic stimulation as developed by Martin (16) in which the threshold of nerve stimulation or the intensity of stimulus necessary to cause physiological response is stated in what he describes as Z units.

The apparatus by which these readings are obtained consists of a calibrated inductorium, a Weston milliammeter, a rheostat, a battery, and a key. A vibrating reed interrupter with an interruption rate of 10 per second maintained by a separate battery is placed in the primary circuit. The current of the primary circuit has a voltage of about 2 and amperage of 0.3.

The inductorium had been previously calibrated,[1] the calibration depending upon the principle that "the physiological intensities of break induction shocks are proportional, other factors remaining constant, to the expression $\frac{MI}{L}$ where M is the mutual inductance between the primary and secondary coils, I the intensity of the primary current, and L the self-inductance of the secondary coil" (17). The expression for stimulation value is, therefore, $Z = \frac{M}{L} I$, Z being the stimulation value, $\frac{M}{L}$ the calibration value for the particular position of the secondary coil, and I the primary current in amperes.

The majority of the experiments were performed on decerebrated cats,[2] but in some experiments it was necessary to use urethane or ether anesthesia, and a number of parallel observations were made on dogs and rabbits. In all instances, excepting when specifically noted, the experiments were not begun until the animals were in an advanced stage of intoxication.[3]

[1] The calibrations were done by Dr. Martin.

[2] Decerebration was always done under ether anesthesia.

[3] The details of the experiments referred to in this report will be discussed by Shevky in an article entitled, "A study of the physiological action of the toxin of *Clostridium botulinum*."

EXPERIMENTAL.

I. Threshold of Stimulation of the Vagus Nerves in Normal and in Botulism Animals.

A. For Cardiac Inhibition.

1. In the first group of experiments the tonic effect upon cardiac muscle with slowing of the heart rate was taken as the index of excitability of the vagus nerves. Both vagi were cut at as high a level as possible and the distal ends were stimulated singly or simultaneously through Sherrington electrodes. Coincident blood pressure records were obtained through a cannula inserted into the left carotid artery and connected to a Huerthle manometer.

The method of obtaining the threshold of stimulation may be briefly described as follows: A stimulus of so low an intensity that it will not induce a slowing of the heart rate is first applied, and then by gradually approaching the primary coil of the inductorium with the secondary, stimuli of successively increasing intensities are applied until a point is reached at which the physiological effect of slowing the heart rate is first observed. The point upon the calibrated inductorium at which this impulse occurs is taken as the index of the threshold of stimulation for initial physiological response, and, by means of the formula which was devised by Martin, is expressed in terms of Z units. After the threshold for initial response has been obtained the secondary coil is then moved backwards, away from the primary coil, in short steps until the point is reached beyond which no further retardation of the heart rate can be detected. From this reading is calculated the threshold of the minimum effective stimulus.

In the study of normal animals it was found that the threshold of stimulation of the vagus nerve for initial cardiac inhibition in ten cats lay between 37 Z and 406 Z with an average of 172.1 Z, in three rabbits between 89 Z and 277 Z with an average of 164.3 Z, and in one dog was 100 Z, whereas in eleven botulism cats the readings ranged between 168 Z and 2,250 Z with an average of 1,098.2 Z, in three rabbits between 1,720 Z and 2,620 Z with an average of 2,113.3 Z, and in two dogs they were 614 Z and 1,150 Z respectively (Table I).

The experiments showed that in normal as well as in intoxicated animals it requires a higher intensity of stimulus to induce the initial physiological response (slowing of the heart rate) than is necessary to repeat the phenomenon within a short interval after the initial retardation has been effected. In Text-fig. 1, *A* is shown the record of a normal cat in which a stimulus of 192 Z was required to induce the first physiological response, although, successively, 168 Z, 129 Z, and 100 Z were sufficient to repeat the retardation; and in Text-fig.

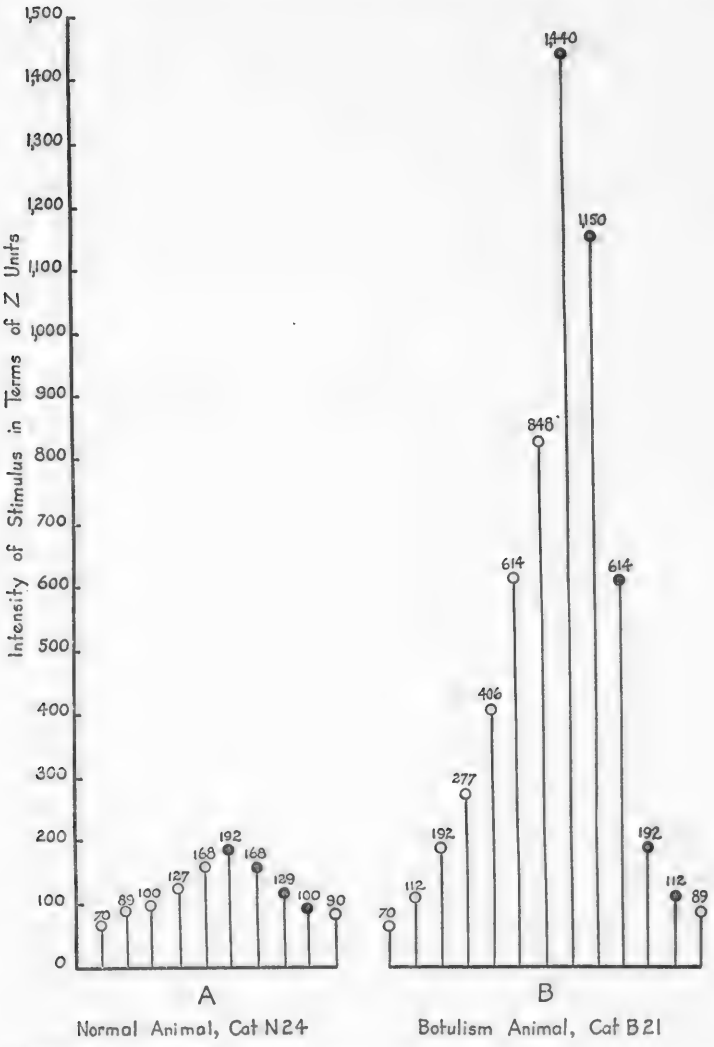
TABLE I.

Threshold of Stimulation of the Vagus Nerves for Initial Retardation of the Heart Beats.

Normal animals.			Animals with *botulinus* intoxication.		
Animal No.	Threshold in terms of Z units.	Nerve stimulated.	Animal No.	Threshold in terms of Z units.	Nerve stimulated.
Cat N18	37	Left.	Cat B22	277	Right.
	50	Right.	" B11	469	Left and right.
" N22	60	Left.	" B10	490	" " "
" N20	64	Right.	" B20	848	" " "
" N27	89	Left and right.	" B36	1,000	" " "
" N30	127	" " "	" B9	1,150	" " "
" N19	192	Right.	" B34	1,250	" " "
" N24	192	Left and right.	" B30	1,295	Right.
" N16	277	" " "	" B21	1,440	"
" N23	277	" " "	" B37	1,720	Left and right.
" N31	406	" " "	" B31	2,250	" " "
Rabbit N1	89	" " "	Rabbit B8	1,720	" " "
" N2	127	" " "	" B4	2,050	" " "
" N4	277	" " "	" B5	2,620	" " "
Dog N1	100	" " "	Dog B1	1,150	" " "
			" B2	614	" " "

1, *B* is the record of a botulism cat in which it required a stimulus of 1,440 Z to induce initial slowing of the heart, although after the initial response had been induced it could be repeated by a stimulus of as low as 112 Z.

In the botulism animals it was found that although the initial threshold of stimulation was always high, the secondary or final threshold of minimum effective stimulus in many instances lay within the limits of variation for normal animals.



° indicates no response.

• indicates slowing of the heart rate.

TEXT-FIG. 1. Stimulation of the vagus nerves to induce slowing of the heart rate.

2. In order to determine whether there is any correlation between the extent of blocking of the impulse in the vagus nerves and the degree of *botulinus* intoxication, a series of eight cats of as nearly identical weight and size as possible was injected with *botulinus* toxin in sufficient quantity to cause death in approximately 48 hours. One animal was sacrificed every 6 hours after the injection so that the vagus nerve tests were recorded at approximately 6, 12, 18, 24, 30, 36, 42, and 46 hours after the administration of the toxin. Only the animals which were allowed to survive for more than 30 hours showed clinical signs of intense intoxication.

TABLE II.

*Intensity of Vagus Nerve Stimulus Necessary to Cause Slowing of the Heart Rate at Stated Intervals after the Administration of botulinus Toxin.**

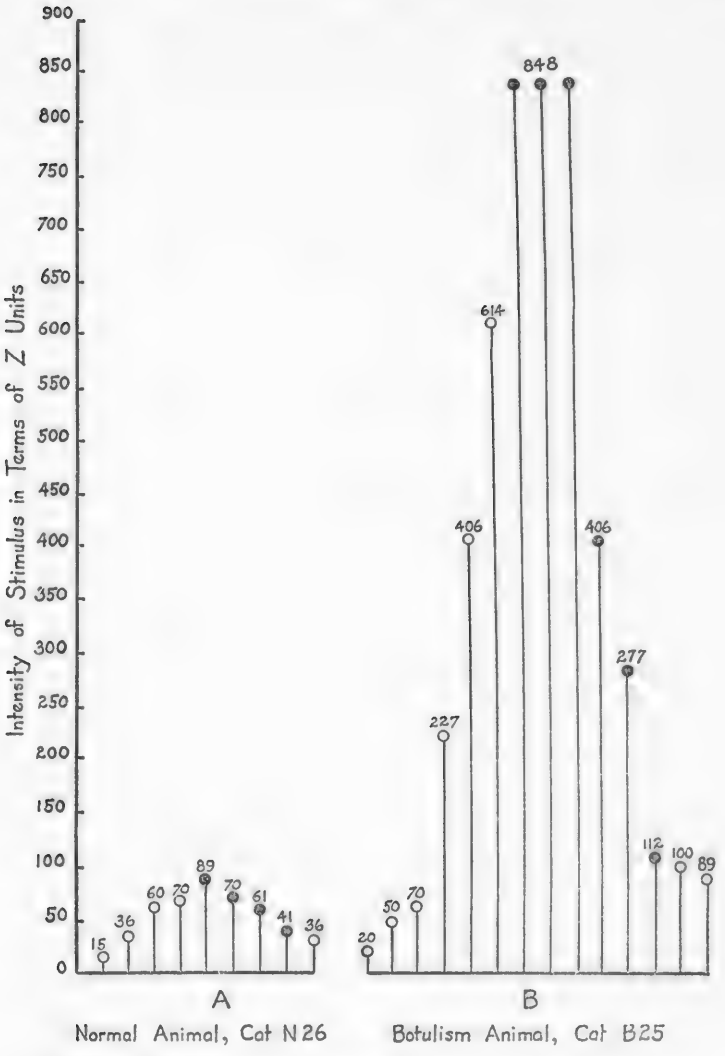
Animal No.	Weight.	Length of time after injection.	Threshold in terms of Z units.
	gm.	*hrs.*	
Cat B25	2,340	6	98
" B27	2,200	12	127
" B29	2,400	18	100
" B32	2,310	24	149
" B33	2,420	30	355
" B34	2,210	36	1,250
" B36	2,300	42	1,000
" B37	2,320	46	1,720

* Each animal received the same amount of toxin per kilo of weight, the total dose being that which should cause death in approximately 48 hours.

The results of these experiments are shown in Table II. The animals which survived for 30 hours or less and which did not show clinical signs of *botulinus* intoxication showed vagus readings which were within the limits of variation for normal animals, although those of the 30 hour cat approached the upper limit of normal; but the three animals which were clinically ill and which survived for 36, 42, and 46 hours, respectively, gave vagus readings which were much higher than any observed under normal conditions.

B. For the Augmentor Effect upon the Small Intestine.

Cats were used in these experiments. After decerebration a cannula was inserted into the trachea for artificial respiration, the splanch-



○ indicates no response.

● indicates augmentation of intestinal movements.

TEXT-FIG. 2. Stimulation of the vagus nerves to augment movements of the small intestine.

nic nerves were cut within the thorax, and the animals were immersed in normal salt solution which was maintained at body temperature. Both vagi were cut and placed in glass-shielded electrodes so that they received simultaneous stimulation. The movements of the exposed intestine were recorded upon a revolving drum by means of a lever which was attached to the intestine by a fine silk thread.

The method of determining the threshold values was the same as that described for the study of the vagus effect upon the heart. A stimulus of low intensity was first applied and the intensity of successive stimuli was gradually increased until the first physiological response was obtained. The coil of the inductorium was then moved back until the minimum intensity of stimulus which would cause augmentation of the intestinal movements was determined.

TABLE III.

Threshold of Stimulation of the Vagus Nerves for Initial Augmentor Effect upon the Small Intestine.

Normal animals.			Animals with *botulinus* intoxication.		
Animal No.	Threshold in terms of Z units.	Nerves stimulated.	Animal No.	Threshold in terms of Z units.	Nerves stimulated.
Cat N25	52	Left and right.	Cat B29	277	Left and right.
" N21	61	" " "	" B23	614	" " "
" N28	64	" " "	" B25	800	" " "
" N26	89	" " "	" B27	1,150	" " "

It was found that there is complete analogy to the effect of stimulation of the vagi upon the heart in that it requires a higher intensity of stimulus to induce initial augmentation of the movements of the small intestine in normal and in botulism animals (Text-fig. 2) and that in *botulinus* intoxication the threshold of stimulation for initial augmentation of the intestinal movements is much higher than in normal animals (Table III).

II. Threshold of Stimulation of the Chorda Tympani Nerve for Initiating the Flow of Saliva.

Decerebrated cats were used in these experiments. Dissections were made to expose the submaxillary and retrolingual ducts and the

lingual nerve with its chorda tympani branch. The lingual nerve was ligated on either side of the chorda tympani and severed beyond each ligature so that a small stump remained by which the small nerve could be manipulated.

The chorda tympani nerve was stimulated through small glass-shielded electrodes and the salivary secretion was measured by its flow through a cannula in the submaxillary duct into a graduated glass tube of small bore which was elevated at an angle of about 30°. The method of varying the intensity of the stimuli was the

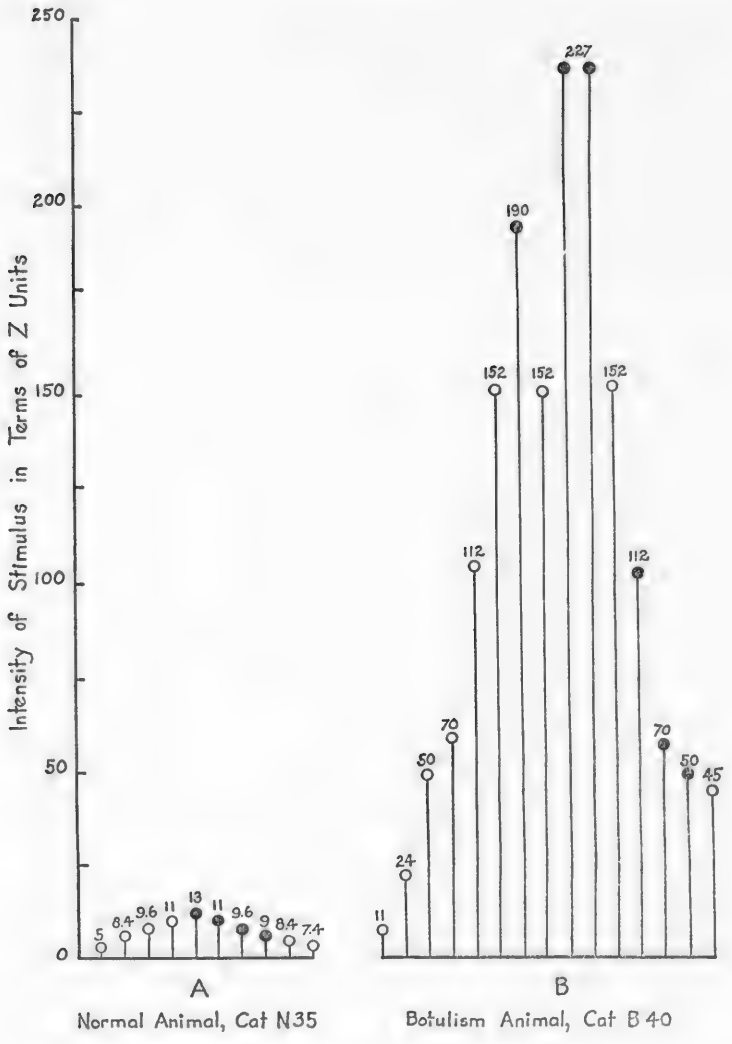
TABLE IV.

Threshold of Stimulation of the Chorda Tympani Nerve for Initiating the Flow of Saliva.

Normal animals.		Animals with *botulinus* intoxication.	
Animal No.	Threshold in terms of Z units.	Animal No.	Threshold in terms of Z units.
Cat N35	13	Cat B38	70
" N26	16.5	" B39	89
" N36	34	" B43	112
" N40	70	" B42	127
		" B40	277
		" B45	340
		" B41	385

same as that employed in the previous experiments, excepting that it was necessary to allow sufficient time to elapse between stimulations to permit the flow of saliva to be recorded. This interval was fixed at 20 seconds.

It was found that in normal animals the initial flow of saliva was induced by stimulation of from 13 Z to 70 Z, whereas in botulism animals from 70 Z to 385 Z were required (Table IV). In the function of this nerve, as in that of the vagus, a lower intensity of stimulus will maintain the salivary flow after it has once been established (Text-fig. 3).



○ indicates no response.

• indicates beginning of salivary secretion.

TEXT-FIG. 3. Stimulation of the chorda tympani nerve to induce a flow of saliva.

III. Threshold of Stimulation of the Pelvic Nerve (Nervus Erigens) for Initiating Movements of the Bladder Wall and Erection of the Penis.

Decerebrated male cats were used in these experiments. The pelvic nerve was exposed and severed as nearly as possible to its point of emergence from beneath the sacrocaudal muscles. It was stimulated through shielded electrodes as in the previous experiments. In the majority of animals examined the erection of the penis and the contractions of the walls of the bladder were found to occur simultaneously.

TABLE V.

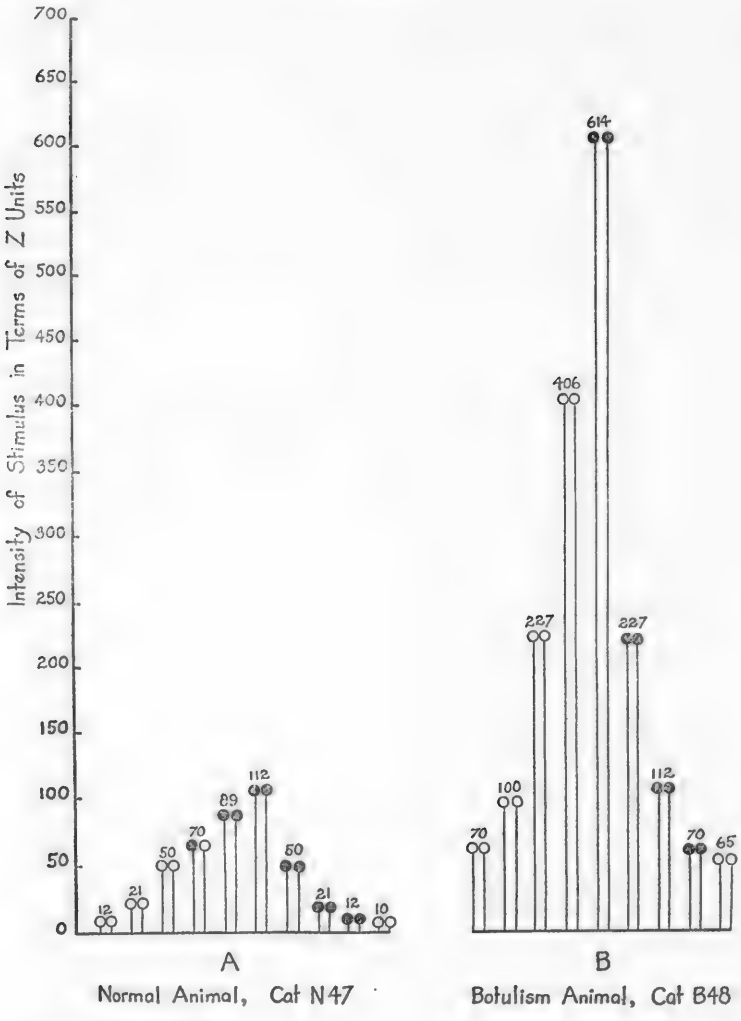
Threshold of Stimulation of the Pelvic Nerve (Nervus Erigens) for Initial Contraction of the Bladder Wall and Erection of the Penis.

Normal animals.			Animals with *botulinus* intoxication.		
Animal No.	Threshold in terms of Z units.		Animal No.	Threshold in terms of Z units.	
	Bladder.	Penis.		Bladder.	Penis.
Cat N46	16	16	Cat B49	488	488
" N42	30	30	" B46	515	515
" N47	89	70	" B48	614	614
" N44	50	66	" B47	614	848
" N45	70	70	" B50	614	848

The experiments showed that in normal cats the first effective stimulus of the nervus erigens is between 16 Z and 70 Z, whereas in botulism animals it was necessary to employ from 488 Z to 848 Z before any physiological effect was produced (Table V). In both normal and botulism animals erection of the penis and movements of the bladder wall could be induced by stimuli of lower intensity after the initial effect had been accomplished (Text-fig. 4).

IV. Threshold of Stimulation of the Oculomotor Nerve for Contraction of the Pupil.

By slightly modifying Sherrington's technique of decerebration it is possible to remove the cerebrum of cats and rabbits and leave the oculomotor nerves intact. The trephine opening may be enlarged sufficiently to allow direct stimulation of the nerves with a small electrode.



° indicates no response.

• indicates physiological response.

TEXT-FIG. 4. Stimulation of the nervus erigens for erection of the penis and inducing contractions of the walls of the bladder. In the chart the first set of results for each animal shows stimulation for erection of the penis, the second set stimulation for inducing contractions of the walls of the bladder.

In our series of experiments it was found that a considerable percentage of cats which were suffering from *botulinus* intoxication, even in advanced stages, failed to show any deviation from normal in the pupillary reflex to light. In the tests of the oculomotor nerve function, therefore, it was necessary to make comparative observations upon normal cats, cats under *botulinus* intoxication in which the pupillary reflex was intact, and those in which the pupillary reflex to light was lost.

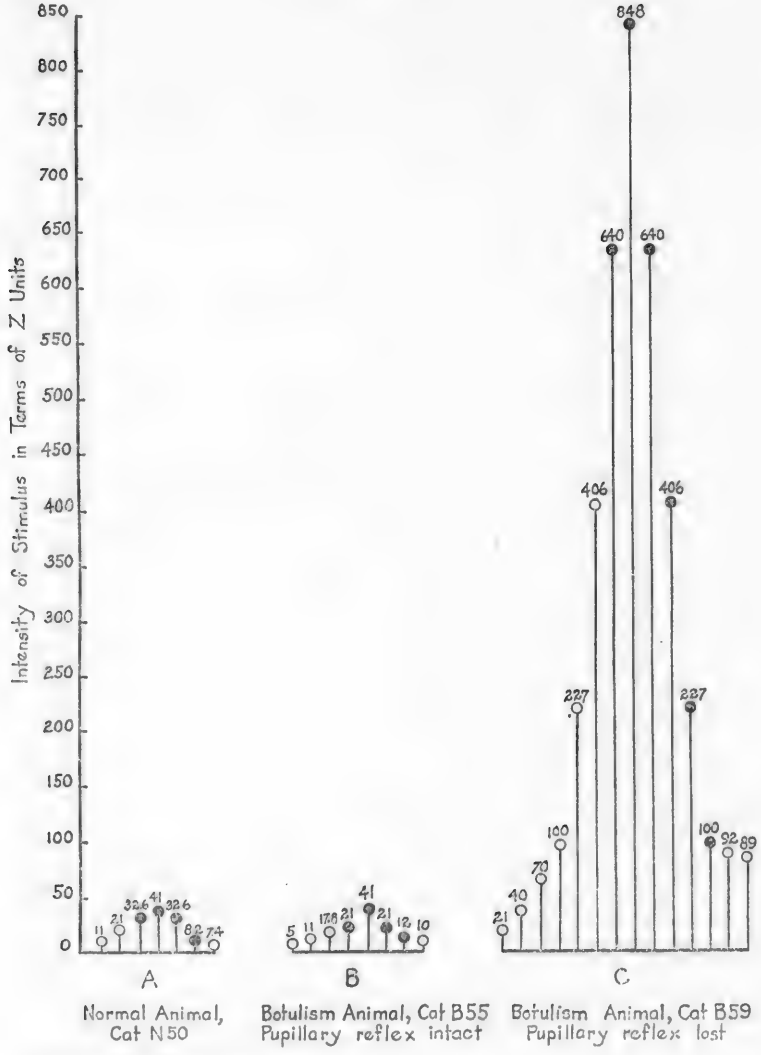
The experiments showed that the same relation between the first effective stimulus and the subsequent minimum effective stimulus

TABLE VI.

Threshold of Stimulation of the Oculomotor Nerve for Initial Contraction of the Pupil.

Normal animals.		Animals with *botulinus* intoxication.			
		Those in which reflex to light is intact.		Those in which reflex to light is lost.	
Animal No.	Threshold in terms of Z units.	Animal No.	Threshold in terms of Z units.	Animal No.	Threshold in terms of Z units.
Cat N58	5.7	Cat B65	7	Cat B64	280
" N62	6.3	" B63	9	" B67	333
" N61	8	" B57	10	" B53	383
" N52	9	" B74	10	" B66	383
" N56	11.6	" B55	21	" B71	614
" N54	13.6			" B59	640
" B71	21.6			" B68	848
" N50	32.6				

for pupillary contraction obtains in the oculomotor medullated fibers as in the other autonomic nerves which have been described (Text-fig. 5) and that, although there was practically no difference between the thresholds for initial contraction of the pupils for normal cats and the botulism cats in which the pupillary reflexes were intact, there was a marked increase in the threshold for initial contraction of the pupils in the cats in which the light reflex was lost. The values obtained in normal cats and in those without loss of pupillary reflex lay between 5.7 Z and 32.6 Z, whereas those for botulism cats which had lost the pupillary reflex lay between 280 Z and 848 Z (Table VI).



○ indicates no response.

● indicates contraction of the pupils.

TEXT-FIG. 5. Stimulation of the oculomotor nerve to induce contraction of the pupil.

V. Effect upon the Vasomotor Nerves.

In a considerable number of the experiments blood pressure records were obtained by inserting a cannula into the carotid artery. There was no variation in the blood pressure of botulism animals which lay outside the limits of variation observed in normal animals.

VI. Effect upon the Splanchnic Nerves.

Decerebrated cats were placed upon their backs in a tank containing Ringer's solution which was maintained at body temperature, the abdomen was opened under the solution, and the movements of the intestine were observed and recorded as in the experiments upon the effect of stimulation of the vagus nerves.

It was found that the appearance of the intestines in botulism animals was identical with that described by Bayliss and Starling (18) for normal animals, and which these authors attributed to continuous tonic and reflex influences from the splanchnic nerves. When the splanchnic nerves were cut spontaneous rhythmic movements of the intestine were noted which were identical with those occurring under like conditions in normal animals, and immediately after the death of a botulism animal the same rhythmic movements of the intestine were noted that occur after the death of a normal animal. All these observations indicate that in *botulinus* intoxication the function of the splanchnic nerves is not lost.

CONCLUSIONS.

A survey of the results of these experiments shows, we believe conclusively, that in *botulinus* intoxication in cats, dogs, and rabbits there is a specific effect upon the portions of the autonomic nervous system which Gaskell (14) described as the bulbosacral and prosomatic outflows of connector fibers respectively, which results in a blocking of the nerve impulses of these nerves. The experimental as well as the clinical evidence indicates that there is no damage to the nerves of the thoracicolumbar outflow.

The exact location of the damage has not been ascertained nor has the mechanism by which the nerve impulse is blocked been determined. The experiments show, however, that the lesions in these

portions of the nervous system are not of central distribution but are peripheral, and that the block cannot be due to an organic break in the conduction apparatus but must be due to some derangement which is relatively unstable. If it were otherwise it would not be possible to induce a physiological response even by massive stimulation, nor could the response be subsequently repeated by stimuli which lie within the limits of normal intensity.

The application of the results of these experiments to the clinical manifestations of botulism will be discussed in a later report after the effect of the toxin upon the skeletal motor nerves has been described.

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